MOLECULAR INTERACTIONS OF THE *Streptococcus mutans* SURFACE PROTEIN P1: CONTRIBUTIONS TO PROTEIN STRUCTURE, STABILITY, AND TRANSLOCATION

By

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MOLECULAR INTERACTIONS OF THE *Streptococcus mutans* SURFACE PROTEIN P1: CONTRIBUTIONS TO SURFACE STRUCTURE, STABILITY AND TRANSLOCATION

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May 2005

Chair: L. Jeannine Brady
Major Department: Oral Biology

Streptococcus mutans is considered to be the major etiologic agent of human dental caries. Attachment of *S. mutans* to the tooth surface is required for the development of caries and is mediated, in part, by the 185 kD surface protein variously known as antigen I/II, PAc, and P1. Such proteins are expressed by nearly all species of oral streptococci and have been identified as possible antigens for vaccine development. In addition, researchers are utilizing P1 to study immune response and immunomodulation .

The goal of this research was to identify intramolecular interactions within P1 and to examine their contributions to P1 structure, stability, and translocation. To that end, this research demonstrates a) that several anti-P1 monoclonal antibodies (mAbs) require the simultaneous presence of the alanine-rich and proline-rich regions for binding, b) that the proline-rich region of P1 interacts with the alanine-rich region, c) that like the proline-rich region, the alanine-rich region is required for the stability and translocation

of P1, d) that both the proline-rich and alanine-rich regions are required for secretion of P1 in *E. coli*, and e) that in *E. coli* P1 is secreted in the absence of SecB. Additionally, it was demonstrated that the chaperone RopA (trigger factor) was not required for P1 translocation. However, its absence resulted in reduced P1 mediated adherence to salivary agglutinin, suggesting a role in P1 maturation. DnaK was also shown to be involved in P1 translocation and *dnaK* mRNA levels were affected by the presence of P1 deletion constructs. Furthermore, the A- and P-regions of P1 were shown to be capable of interacting with the A- and P-regions of the Antigen I/II proteins, SspA and SspB, from *Streptococcus gordonii.* These interactions restored epitopes recognized by several anti-P1 mAbs. Replacing the A-region of P1 with the A-regions of SspA and SspB also restored some mAb binding, but did not restore stability and translocation of P1 to the cell surface. The results of this research have implications for understanding surface localization of virulence factors in pathogenic microorganisms and for understanding how the protein structure of a vaccine antigen contributes to recognition by antibodies.

CHAPTER 1
INTRODUCTION

Streptococcus mutans and Dental Caries

Streptococcus mutans is considered to be the major etiological agent of human dental caries [1, 2], one of the most common infectious diseases that affect humans. *S. mutans* was first identified in a human carious lesion in 1924 and isolates were later divided into eight serotypes designated *a* through *h* based on differences in cell wall carbohydrate composition [3, 4]. Concurrent DNA hybridization studies further categorized the serotypes as four genetic types based upon the guanine plus cytosine (G+C) content of their genomes [5, 6]. The four genetic types were subsequently classified as different species, *Streptococcus critus* (serotype *a*), *Streptococcus rattus* (serotype *b*), *Streptococcus sobrinus* (serotypes *d, g, h*), and *S. mutans* (serotypes *c, e, f*). These species are collectively known as mutans streptococci [7]. *S. mutans* serotype *c* is the most common mutans streptococcus isolated from human dental plaque [1, 2].

S. mutans is equipped with several proteins that enable its attachment and subsequent colonization of the tooth surface. In the presence of sucrose, extracellular glucosyltransferases (GTF) synthesize several forms of branched extracellular glucans. These glucans provide a matrix for the aggregation of *S. mutans* and other oral streptococci through interaction with proteins such as the bacterial surface-localized glucan-binding proteins (GBP). *S. mutans* possesses four GTF genes, *gtf*A [8], *gtf*B [9], *gtf*C [10], and *gtf*D [11], and three GBP genes, *gbp*A [12], *gbp*B [13], and *gbp*C [14]. Mutational inactivation of the GTF genes has shown that their products are important to

cariogenecity. However, a model for colonization of the tooth surface by *S. mutans* suggests that initial attachment to the tooth pellicle is protein-mediated followed by glucan-dependent bacterial accumulation [15]. The surface proteins that are implicated in the initial adherence of *S. mutans* are members of the antigen I/II super-family of multifunctional adhesins and are variously known as antigen I/II [16], Ag B [17], IF [18], P1 [19], SR [20], and PAc [21], and are encoded by the genes *spaP* or *pac*. Antigen I/II-like molecules are expressed in nearly all of the oral streptococci [22] and include SpaA [23]and PAg [24] from *S. sobrinus*, SSP-5 from *Streptococcus sanguis* [25], and SspA [26] and SspB [27] from *Streptococcus gordonii.*

Major Surface Protein P1

The genes *spaP* and *pac* have been cloned and sequenced [21, 28, 29]. N-terminal amino acid sequencing of the proteins and the predicted amino acid sequences indicate that the *spaP* and *pac* gene products differ by only 36 residues. Major characteristics of the M_R~185,000 P1 include a 38 residue amino-terminal signal sequence, a region containing three 82-residue alanine-rich repeats, a 150 residue variable region in which 20 of the 36 aforementioned amino acid substitutions reside, a central proline-rich region containing three 39-residue tandem repeats, carboxy-terminal wall- and membrane-spanning regions, and an LPXTG wall anchor motif [28] (Fig. 1). Secondary structure predictions of P1 based upon the sequence of *spaP* indicate that the alanine-rich region would form an α-helicle coiled-coil structure while the central proline-rich region would form an extended β-sheet structure [28]. Recently the variable region of the *S.mutans* serotype *f* antigen I/II was subcloned, and its crystal structure determined. The crystal structure data indicate that the variable region forms a flexible β-sandwich [30].

Figure 1. Schematic representation of P1

Antigen I/II polypeptides are structurally complex and exhibit diverse binding properties, which mediate interactions with a variety of substrates including host salivary agglutinin, fibronectin, fibrinogen, and collagen [31, 32]. Several regions have been implicated in the binding activities of antigen I/II polypeptides. Brady et al [33] provided evidence that P1 possessed multiple sites contributing to salivary agglutinin binding and that different regions might be involved in binding to soluble or immobilized salivary agglutinin. Later, Scatchard analysis of antigen I/II binding to saliva-coated hydroxyapetite showed the binding to be mediated by two sites [34]. Investigators have showed that recombinant peptide fragments derived from the A-region bound salivary agglutinin [35] or salivary glycoproteins [36] and Senpuku et al. (1995) demonstrated that antibodies specific to a peptide fragment derived from PAc aa residues 200-481 inhibited the binding of fluid-phase salivary components to immobilized PAc. Furthermore, an antigen I/II peptide fragment consisting of a.a. residues 816-1213 blocked *S. mutans* cell adhesion to saliva-coated hydroxyapatite [37] and Kelly et al. [38] found antigen I/II derived peptides consisting of residues 1005-1044 and 1085-1114 to be inhibitory to *S. mutans* adhesion to salivary glycoproteins.

Proline and Proline-Rich Regions

Proline is unique among the amino acids in that its side chain is covalently bound to the backbone amide. As a result of this unusual bond, the proline residue has a restricted backbone conformation [39], the bulkiness of its side chain restricts the conformation of the preceding residue [40], and the proline is unable to act as a hydrogen bond donor. Proline is recognized as an α-helix and β-sheet breaker and is often located one or two residues C-terminal of an α-helix [41]. A sequence of four or more prolines in a row adopts the conformation of an extended structure with three residues per turn, known as a polyproline II helix [42]. The polyproline II helix is a major structural element in collagen, pancreatic polypeptides, and neuropeptides [43]. In a survey of surface proteins, there were 90 occurrences of polyproline II helixes in 80 non-homologous proteins [44].

Proline-rich regions are biologically important in numerous unrelated proteins in a variety of organisms, both eukaryotic and prokaryotic. Although the functions of proteins containing proline-rich regions can be quite diverse, the roles of the proline-rich regions appear to be fairly conserved: protein-protein interaction, folding, and structure. In eukarotes, anandamide amidase is responsible for the hydrolysis of neuromodulatory fatty acid amides and esters [45]. The enzyme contains a nine residue proline-rich region, which upon removal resulted in loss of enzymatic activity and a change in subcellular localization of the enzyme. It is suggested that the proline-rich region may be essential for the correct folding of the amidase, although similar proline-rich regions are not found in non-mammalian amidases [46]. Involvement in cellular localization has also been demonstrated for the proline-rich region in the eukaryotic enzyme dynamin.

Okamoto et al. has shown that a proline-rich domain is involved in the enzyme's localization to coated pits and in its GTP-binding activity [47].

The relevance of proline-rich domains to the field of medicine can be seen in the human disease Liddle syndrome. Liddle syndrome is a disease of the amiloride-sensitive epithelial sodium channel [48]. The sodium channel is composed of three subunits, two of which contain highly conserved C-terminal proline-rich domains. Frameshift mutations resulting in the deletion of at least 45 residues from the C-terminal segments of the subunits have been the identified causes of this disease. More recently, a missense mutation that results in the substitution of a leucine for a proline (P616) in the conserved proline-rich domain of subunit β has been identified and correlated with the disease. The substitution has greater effect on channel activity than the deletion of the complete C-terminal segment of both the β and γ subunits. It is suggested that this proline residue is involved in an essential interaction with another protein and possibly another subunit [49].

Proline-rich regions are also involved in protein-protein interactions between organisms. In the world of virology, the transformation of primary B lymphocytes with Epstein-Barr virus (EBV) is known to be dependent upon the expression of the EBV nuclear protein 2 (EBNA2) [50]. The 483-residue EBNA2 contains a 36 residue proline-rich region in the N-terminal third of the protein. Of the 230 residues of the N-terminal half of EBNA2, 222 were not essential for transformation of B lymphocytes. The eight essential residues are seven prolines and a glutamine, and it is suggested that they may constitute a critical domain for structure or intramolecular interaction [51]. Lastly, an interaction between a proline-rich insect peptide and a molecular chaperone in bacteria

has been identified. Pyrrhocoricin, an antibacterial peptide originally isolated from the European sap-sucking bug *Pyrrhocoris apterus* [52], kills sensitive species by binding to the bacterial DnaK [53].

Previously, in an attempt to define a role for the P-region in the adhesive function of P1, an internal deletion, P1ΔP (Δ826-996), was constructed [54]. The proline-rich region (P-region) is highly conserved among the antigen I/II family of oral streptococcal proteins, and similar highly repetitive proline-rich sequences have been identified in a wide variety of bacterial proteins [55-64]. Homology to the P-region of P1 is found in numerous surface proteins in both prokaryotes and eukaryotes. Among these are the fibronectin binding proteins of *Streptococcus pyogenes* and *Staphylococcus aureus* [65, 66], an immunogenic secreted protein (*isp*) of *S. pyogenes* [67], and the virulence associated suface protein, PspA, of *S. pneumonia* [68]. The internally deleted polypeptide P1ΔP was expressed in both *E. coli* and in *S.mutans* PC3370, an isogenic *spaP*-negative mutant. Western blots of P1ΔP expressed in *E. coli* revealed a loss in reactivity for fiveof eleven P1-specific MAbs. These five mAbs also did not react to a subclone of the P-region (826-996), suggesting that they recognize a complex P1 epitope that is dependent upon the presence of the P-region. Although P1ΔP contains the signal sequence, it was not translocated to the surface of *S. mutans* PC3370 (*spaP*). Also, in comparison to full-length P1 expressed from pDL289, only low levels of P1ΔP were detected in the cytoplasm of PC3370, while mRNA levels were equivalent. These data suggest that the P-region may be required for P1 stability and subsequent translocation to the cell surface.

Protein Translocation

Since P1ΔP possessed the N-terminal signal sequence and C-terminal cell wall anchor motif, the lack of P1ΔP expression on the cell surface was unexpected. Proline-rich regions are known to be involved in a variety of intra- and intermolecular protein-protein interactions [69-78], including chaperone-like activities. Wang et al. [79] identified a centrally located proline-rich region in the serine protease, Factor C.

The *Limulus polyphemus* (horseshoe crab) Factor C is a 132-kDa secreted serine protease and contains a centrally located proline-rich region. The role of the proline-rich region in the secretion and function of Factor C was investigated through the construction and expression of homologues with and without the proline-rich region. The proline-rich region is flanked by an amino-terminal lectin binding domain and a carboxy-terminal protease domain. Factor C is 1019 amino acids in length, and the proline-rich region spans residues 630 – 690. Deletion of either the lectin binding or protease domains resulted in peptides that were stably expressed and secreted by the cell. In contrast, deleting the proline-rich region resulted in a protein that was found in the cytoplasm but was no longer secreted. In addition, expression of a truncated peptide consisting of the amino-terminal 329 residues of Factor C was stable and secreted while a peptide consisting of the secretion signal fused to the carboxy-terminal protease domain was not secreted. Interestingly, the addition of the proline-rich region amino-terminal to the protease domain restored secretion of the peptide, but fusing the proline-rich region carboxy-terminal to the protease domain did not restore secretion.

To establish a role for the proline-rich region in the folding of Factor C, a partial trypsin digestion analysis was performed. Trypsin cleaves preferentially at unfolded regions in proteins, and although there are nearly 100 potential trypsin cleavage sites in

Factor C, four bands were detected after partial digestion. The visible fragments were attributed to compactly folded domains. However, Factor C without the proline-rich region was not detectable after 10 minutes of trypsin digestion indicating that the protein was in an unfolded trypsin-susceptible conformation. This study revealed that the proline-rich region is essential for the stability and secretion of Factor C. Because this effect was similar to that previously described for the P-region of P1 [54] these authors suggested that internal proline-rich regions may act as intramolecular chaperones for correct folding and secretion of proteins that contain them. The homology of the P-region of P1 to the surface proteins of numerous organisms and its conservation within oral streptococci suggest that it plays an important role in P1, and considering the prevalence of proline-rich regions in protein-protein interactions, it is likely that the P-region is involved in such an interaction.

Recently, Van Dollarweed et al. (2003) demonstrated that the P-region of P1 binds to a polypeptide fragment of P1 that contains the alanine-rich region (A-region). This interaction restored the reactivity of a P1 specific Mab that was not reactive to either of the fragments individually and suggests that these regions interact in mature, surface expressed, P1. X-ray crystallography has revealed that the variable region of P1 forms a flexible beta-sandwich that would place the P-region and A-region into close proximity [30]. Given that the P-region is required for the native structure, stability, and translocation of P1 and that the P-region interacts with a fragment of P1 containing the A-region, it is likely that the A-region may also play a role in the structure, stability, and translocation of P1.

To fully elucidate the role of the P-region in P1 translocation, a better understanding of the molecule's route of translocation represents an important goal. There is no experimental data that identify the secretion pathway P1 or antigen I/II-like proteins use. However, based upon the method of P1 cell wall anchoring, a route of translocation has been predicted. Gram-positive surface proteins containing the conserved C-terminal LPXTG motif, such as P1, are anchored to the cell wall by the membrane anchored transpeptidase, sortase. During cell wall anchoring, sortase cleaves surface proteins between the threonine and the glycine of the LPXTG motif [80]. Following cleavage in *S. aureus* and *Listeria monocytogenes*, the proteins are linked to cell wall peptides via an amide bond [81, 82]. Although several aspects of peptidoglycan structure in Gram-positive bacteria are variable [83], the principles of surface protein anchoring appear to be conserved [84]. Lee and Boran[85] identified and insertionally inactivated the gene encoding sortase, *srtA*, in *S. mutans*. As predicted, SrtA$^-$ mutants secreted P1 into the supernatant demonstrating that P1 is indeed a sortase anchored protein. Current evidence suggests that sortase anchored proteins are translocated via the Sec translocase [86].

The Sec-dependent secretion pathway has been well characterized and studied in *E. coli* and to a lesser extent in *B. subtilis*. In *E. coli*, the Sec-translocase consists of SecA, SecY, SecE, SecG, SecD, SecF, and YajC [87]. Two major targeting pathways converge on the Sec-translocase, the signal recognition particle (SRP) pathway and the SecB pathway. The *E. coli* SRP consists of a 4.5s RNA and the GTPase Ffh, both of which are required for cell viability [88]. Signal peptides of nascent polypeptides are recognized by the SRP as they emerge from the ribosome [89]. SRP binding stalls translation and

targets the SRP-ribosome complex to the SRP receptor, FtsY [90, 91]. The complex is then targeted to the Sec-translocon where the ribosome docks and translation is restored. The preprotein is cotranslationally translocated across the membrane via an integral membrane complex consisting of SecY, SecE, and SecG. The ATPase SecA provides energy for the translocation [87]. In the case of posttranslational secretion, the cytoplasmic chaperone SecB targets preproteins to the Sec-translocon. SecB binds to nascent and full-length preproteins as they emerge from the ribosome [92]. SecB interaction prevents premature folding of the preprotein and delivers it to the Sec-translocon in a secretion-competent state. Binding of the SecB-preprotein complex with SecA results in the transfer of the preprotein to SecA and the release of SecB [93]. The preprotein is subsequently translocated across the membrane through the Sec-translocon [94].

The SRP pathway exists in both gram-negative and gram-positive bacteria. Identified homologs of the Sec-dependent pathway components in *B. subtilis* include SecA, SecYEG, SecDF, YrbF, Ffh, and scRNA. As the genome sequences of gram-positive bacteria have become available, investigators have searched for homologs of SecB to no avail. However, a *B. subtilis* complementation study of an *E. coli* SecB null mutant revealed a functional ortholog, CsaA, with partially overlapping binding characteristics [95-97]. As previously stated, the SRP is essential for viability in *E. coli*, and this was assumed to be the case in all organisms. However, an Ffh null mutant in *S. mutans* is viable, and P1 is translocated and expressed on the cell surface [98]. This suggests that if P1 secretion is Sec-dependent, the targeting pathway is likely to be SecB-

like and may require a SecB ortholog or possibly an unrelated chaperone with similar functions.

DnaK and RopA

The translocation of P1 to the cell surface in an *S. mutans* mutant devoid of the SRP pathway [98] would suggest that P1 is post-translationally secreted, and a protein of 1561 residues would most certainly require interactions with chaperones to prevent misfolding and aggregation while transiting the cytosol. The 70-kD heat shock proteins (Hsp70s) are ubiquitous proteins found in the bacterial cytosol and several compartments of eukaryotic cells including the endoplasmic reticulum, the mitochondria, and the cytosol [99]. The *E. coli* Hsp70, DnaK, has been extensively studied and is involved in a variety of cellular processes, including both protein folding and degradation. In studies of substrate specificity it has been shown that DnaK preferentially binds to peptides that contain hydrophobic residues [100]. In proteins these hydrophobic residues are typically found in the core of the folded structure, or in subunit interfaces [101]. Nascent polypeptides emerging from the ribosomes, as well as malfolded proteins, display short hydrophobic regions that are not exposed in the protein's native conformation. DnaK binds to these exposed hydrophobic segments, thereby preventing aggregation and further misfolding.

Another chaperone that interacts with nascent polypeptides is trigger factor, a ribosome-associated peptidyl-prolyl *cis-trans* isomerase (PPIase). In *S. mutans* trigger factor is known as RopA. Trigger factor associates with the large ribosomal subunit at the peptide exit channel and binds to nearly all nascent polypeptides [102]. There is evidence that trigger factor cooperates with DnaK to promote the folding of a variety of cytosolic *E. coli* proteins [103] and that they share substrates and binding specificities

[104]. In fact, DnaK is not recruited to translating ribosomes that lack trigger factor [105]. Besides its chaperone activities, trigger factor can catalyze *cis-trans* isomerizaion of peptidyl-prolyl peptide bonds. The PPIase activity of trigger factor is not required by all of the proteins that require trigger factor for proper folding, however the PPIase activity is essential for some [106]. In *S. pyogenes*, the PPIase activity of trigger factor influences the conformation of the nascent cysteine protease, SpeB, which in turn directs the protease into one of several alternative folding pathways[107]. The malfolded proteases are subsequently not targeted to the secretion pathway.

Summary and Specific Aims

In an effort to characterize the role of the proline-rich region of P1 in the adherence properties of the molecule, Brady et al. (1998) deleted the region from P1 (P1ΔP). While P1ΔP retained the sequences believed sufficient for expression and translocation, unexpectedly, it was unstable and not translocated to the cell surface. As there is a lack of research regarding protein translocation in gram-positive organisms and Streptococcus in particular, it was of interest to identify the role of the proline-rich region in P1 stability and translocation. Since proline-rich regions are known to be involved in intra- and intermolecular protein-protein interactions, the first specific aim of this work was to identify regions within P1 that interact with the proline-rich region. Once a proline-rich region interaction was discovered, the second specific aim of this study was to analyze the role of the interacting region in the structure, stability and translocation of P1.

P1 is a large and structurally complex molecule as is evident by the change in antibody reactivity seen against P1ΔP that suggests complex and possibly conformational epitopes. Further evidence of the structural complexity of P1 was revealed in the solved

crystal structure of the variable region [30]. Based upon the surface expression of P1 in *S. mutans* lacking the SRP pathway [98] and the presumption that sortase-anchored proteins are secreted via the Sec translocase, the successful post-translational translocation of a large and complex molecule, such as P1, must be dependent upon chaperones. The final specific aim of this work was to examine whether the chaperones DnaK and RopA contributed to P1 translocation or function.

CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains used in this study are listed in Table 1, and all plasmids used are listed in Table 2. Unless otherwise noted, all *S. mutans* strains were grown under anaerobic conditions at 37°C in Todd-Hewitt broth (BBL, Cockeysville, Md.) supplemented with 0.3% yeast extract (THBYE) and kanamyacin (500 μg/ml) as needed. *E. coli* strains were grown aerobically at 37°C with vigorous shaking in Luria-Bertani broth (LB) (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl, pH 7.0) supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) as appropriate. *E. coli* strains MC4100 and CK1953 were grown aerobically at 37°C with vigorous shaking in M9 medium (0.625% [wt/vol] Na_2HPO_4, 0.075% [wt/vol] KH_2PO_4, 0.2% [wt/vol] NaCl, 0.028% [wt/vol] $MgSO_4$, 0.1% [wt/vol] $(NH_4)_2SO_4$, 1% glucose) supplemented with kanamycin (50 μg/ml) and ampicillin (100 μg/ml) as appropriate.

Identification of an Intramolecular Interaction Involving the Proline-Rich Region of P1

Purification of A-region and P-Region-MBP Fusion Proteins

Overnight cultures of *E. coli* harboring pMA3 [54] or pMA41 [35] (Table 1) were diluted 1:100 into fresh Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin and grown to an OD_{600} of 0.5. The medium was supplemented with 0.3 mM isopropyl-b-D-thiogalactopyranoside (IPTG), and the culture was incubated for an additional 2 hours at 37°C . Periplasmic contents were extracted by osmotic shock [108]. Affinity

Table 1. Bacterial Strains

Strain	Description	Source or Reference
E. coli		
DH5α	F^-φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR, recA1 endA1 hsdR17(r_k^- m_k^+ phoA supE44 λ^- thi-1 gyrA96 relA1	
BL21	F^- ompT hsdSB (rb^- mB^-) gal dcm	
MC4100	*E. coli* F^- araD 139Δ, ($argF^-$ lac) U169, relA, rspP, thiA	[109]
CK1953		
S. mutans	MC4100, secB::Tn5	[109]
NG8	Wild-type serotype *c*	[110]
UA159	Wild-type serotype *c*	[111]
PC3370	*spaP*-negative mutant derived from *S. mutans* NG8	[54]
PC3370A	PC3370 transformed with pDL289	[54]
PC3370B	PC3370 transformed with pMAJJ8	[54]
PC3370C	PC3370 transformed with pMAD	[54]
PC3370D	PC3370 transformed with pTS21	(This study)
SM12	UA159 minimal expression of *dnaK*	(Courtesy of J. Lemos, unpublished)
TW90	UA159 (*ropA*)	[112]
S. gordonii M5	Wild-type	[113]

Table 2. Plasmids

Plasmid	Description	Source or Reference
pCR2.1-TOPO	T/A cloning vector	(Invitrogen Corp., San Diego, CA)
pMal-p	Vector for expression of maltose binding protein fusions	(NEB, Beverly, MA)
pMA3	pMal-p derived plasmid containing PCR-amplified DNA encoding amino acids 819 to 1017 of P1	[54]
pMA41	pMal-p-derived plasmid containing PCR-amplified DNA encoding amino acids 186 of 469 of P1	[54]
pDL289	*E. coli*-streptococcal shuttle vector provided by D. le Blanc	[114]
pMAJJ8	pDL289-derived plasmid containing internally deleted *spaP* encoding amino acids 1 to 825 and 997 to 1561	[54]
pDC20	pUC18-derived plasmid containing PCR-amplified *spaP* encoding full-length P1	[54]
pMAD	pDL289-derived plasmid containing PCR-amplified *spaP* encoding full-length P1	[54]
pGEX-4T-2	Vector for expression of glutathione S-transferase fusions	(Amersham Biosciences)
pGEX-A	pGEX-4T-2 derived plasmid containing PCR-amplified DNA encoding amino acids 179-466 of P1	(This study)
pGEX-P	pGEX-4T-2 derived plasmid containing PCR-amplified DNA encoding amino acids 816-1016 of P1	(This study)
pGEX-AP	pGEX-4T-2 derived plasmid containing PCR-amplified DNA encoding amino acids	(This study)
pGEX-BP	pUC18-derived plasmid containing internally deleted *spaP* encoding amino acids 1 to 178 and 465 to 1561	[115]
pTS21	pDL289-derived plasmid containing internally deleted encoding amino acids 1 to 178 and 465 to 1561	[115]

Table 2, continued

Plasmid	Description	Source or Reference
pTS22	pTS21 containing *S. gordonii* M5 DNA encoding the A-region of SspA	(This study)
pTS23	pTS21 containing *S. gordonii* M5 DNA encoding the A-region of SspB	(This study)
pTS31	pDL289 derived plasmid encoding P1 expressed as discontinuous N-terminal (a.a.1-464) and C-terminal (a.a.465-1561) fragments	(This study)
pTS30	pUC18-derived plasmid containing *spaP* encoding amino acids 1 to 465 and 466 to 1561	(This study)
pTS31	pDL289-derived plasmid containing *spaP* encoding amino acids 1 to 465 and 466 to 1561	(This study)
pAR-A	pGEX-6T-P-derived plasmid containing *S. gordonii* M5 DNA encoding the A-region of SspA	[116]
pAR-B	pGEX-6T-P-derived plasmid containing *S. gordonii* M5 DNA encoding the A-region of SspB	[116]
pDDA	pGEM7-derived plasmid containing *S. gordonii* M5 DNA encoding SspA driven by the SspB promoter region	(D. Demuth, unpublished)
pEB-5	pUC19-derived plasmid containing *S. gordonii* M5 DNA encoding SspB	[25]

Table 3. PCR Primers

Primer	Sequence	Target	Underlined Restriction Site
TS7	5'- GCCGACTATCCAGTTAAGTTAAAGGC-3'	*spaP*	
TS8	5'-GCCATACTGTTCTTTAGTTGCCTG-3'	*spaP*	
TS9K	5'-GCGGTACCGTTGGATAAAGTGTGGAGTTTG-3'	*spaP*	*Kpn* I
TS10K	5'-GCGGTACCGCAGTGCGAAGTACCTTATC-3'	*spaP*	*Kpn* I
TS17	5'-AAACTCGAGTCATTCATTCATTGTTCATCTTCGTATGCCT-3'	*spaP*	
TS18	5'-AAACTCGAGGGAGGAAAAATGGCTTCTATTAAAGCTGCACTG-3'	*spaP*	
TS19	5'-GAAGACTTAAAAGCTCATCAAGC-3'	*sspA*	
TS20	5'-CAACTTTTTCTTATATTTGGCAAGATC-3'	*sspA*	
TS21	5'-AAAGATCTAAAAAGTCATCAAGAAGAAGT-3'	*sspB*	
TS22	5'-GAACTCTTTCTTATATTTGGCAAGATC-3'	*sspB*	
TS24	5'-GGATCCAAAGATATGGCAGCTCATAAAGC-3'	*spaP*	*Bam* HI
TS25	5'-GTCGACGATAAATCTTTTTGATATTTGGCAAGATCTG-3'	*spaP*	*Sal* I
TS28	5'-GGATCCGGTAAAATCCGTGCGGTTAAT-3'	*spaP*	*Bam* HI
TS29	5'-GTCGACGACACCAAAGTTCTGTCAATATTAA-3'	*spaP*	*Sal* I
TS41	5'-GGATCCGGTAAAATTCGTGCGGTCAAC-3'	*sspA*	*Bam* HI
TS42	5'-GTCGACAACCAATGTCCGGTCGATATC-3'	*sspA* and *sspB*	*Sal* I
TS43	5'-GGATCCTCAAACATTAATGCAATTGGGGTTC-3'	*sspB*	*Bam* HI
DNAKS	5'-GGAGATGCTGTTGGCGGTGT-3'	*dnaK*	
DNAKAS	5'-GGAAGTATAACAGCATTCGCTGA-3'	*dnaK*	
16SRVS	5'-ATATCTACGCATTTCACCGC-3'	16S RNA	
16SFWD	5'- GCTCTGGAAACTGTCTGACT-3'	16S RNA	

purification of the fusion proteins was performed by passage of the periplasmic fractions through a column of amylose resin (Bio-Rad) and elution with 10 mM maltose by a standard protocol [108]. Purified fusion proteins were quantified using the bicinchoninic acid (BCA) protein assay kit (Sigma) with bovine serum albumin as the standard.

Enzyme-Linked Immunosorbent Assays (ELISA) to Detect A-region and P-Region Interaction

Binding of the A-region to the P-region was measured by ELISA. Sample wells of Costar High Binding plates (Corning Incorporated, Corning, N.Y.) were coated overnight at 4°C, in triplicate, with 100 µl of 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide and 100 ng of purified maltose binding protein (MBP), A-region-MBP, or P-region-MBP. Coating buffer and unbound antigens were removed from the ELISA plate wells, and unreacted sites were blocked with PBS-Tw and overnight incubation at 4°C. Plates were washed four times with PBS-Tw. Purified A-region-MBP, P-region-MBP, and MBP were two-fold serially diluted in PBS-Tw and added to the wells, beginning at 1000 ng/well. The plates were incubated overnight at 4°C and washed four times with PBS-Tw. A-region-specific MAb 3-8D [35] or rabbit anti-MBP Ab (NEB, Beverley, MA) was added to the wells at a 1:1000 dilution. Plates were washed with PBS-Tw and peroxidase-labeled goat anti-mouse IgG or goat anti-rabbit Ig (Cappell) were added to the wells at a 1:1000 dilution. After washing, 100 µl of 0.01 M phosphate citrate buffer (pH 5.0) containing 0.1 M *o*-phenylenediamine dihydrochloride and 0.012% (vol/vol) hydrogen peroxide were added to each well. Plates were incubated for 30 min at room temperature, and the absorbance at 450 nm was recorded by using an MPM Titertek model 550 ELISA plate reader (Bio-Rad).

Elimination of *spaP* DNA Encoding the A-Region

Fragments of *spaP* both upstream and downstream of the A-region were amplified by polymerase chain reaction (PCR) and subsequently ligated together to create *spaP*ΔA. Fidelity of the reactions was confirmed by restriction and sequence analysis. Forward primer TS9k and reverse primer TS8 were used to amplify *spaP* DNA upstream of the A-region, including the *spaP* promoter. Forward primer TS7 and reverse primer TS10k were used to amplify *spaP* downstream of the A-region. Primers TS9 and TS10 contain engineered *Kpn*I restriction sites. Primers TS7 and TS8 were engineered with single base changes that introduce silent mutations, which upon ligation of the PCR products produce a unique *Sfo*I restriction site. Reactions were carried out in a UNO thermoblock thermocycler (Biometra, Tampa, FL) with plasmid-encoded *spaP*, pDC20 [54] as the template and VENT polymerase (NEB) under the following conditions fo 30 cycles: denaturation at 94°C for 30 seconds, primer annealing at 53°C for 30 seconds, primer extension at 72°C for 1 minute or 3 minutes and 30 seconds; and final extension at 72°C for 7 min. The resulting 727 and 3,568 bp gene fragments were ligated together and cloned into the *Sma*I site of pUC18, creating pTS20, which was introduced into *E. coli* DH5α by electroporation. Clones were screened on LB agar supplemented with 100 μg/mL ampicillin and 0.75 μg/mL X-gal (5-bromo-4 chloro-3 indolyl-β-D-galactopyranoside). White colonies were picked and tested for the presence of *spaP*ΔA insert DNA after alkaline lysis. Sequences of all recombinant constructs were confirmed by the DNA sequencing core (University of Florida).

Evaluation of Antibody Binding to P1ΔA

E. coli DH5α harboring pTS20 or pDC20 were grown for 16 hours at 37°C , harvested by centrifugation, and lysed by boiling for 5 minutes in SDS-sample buffer (4% [wt/vol] sodium dodecyl sulfate [SDS], 2% [vol/vol] 2-mercaptoethanol, 20% [vol/vol] glycerol, 125 mM Tris-HCl [pH 6.8], 0.1 mg of bromophenol blue per ml). Proteins were separated by SDS-polyacrylamide gel electrophoresis on 7.5% (vol/vol) acrylamide preparatory gels by the method of Laemmli [117]. Proteins were electroblotted onto nitrocellulose membrane (Schleichter and Schuell, Keene, N.H.) for 1 h at 100 V by the method of Towbin et al. [118]. Immunoblots were blocked with PBS-Tw and cut into 0.5 cm strips. Strips were incubated with anti-P1 MAbs [119, 120] at dilutions of 1:1000 in individual troughs of an Incutray (Schleichter and Schuell). After washing, strips were incubated in peroxidase-labeled goat anti-mouse IgG (Cappel) and developed with 4-chloro-1-naphthol solution (7 ml of PBS, 1 ml of 4-chloro-1- naphthol [Sigma; 3 mg/ml in ice-cold methanol], and 8 μl of 30% [vol/vol] hydrogen peroxide).

Assessment of Epitope Restoration by ELISA

Sample wells of Costar High Binding plates were coated as before with 500 ng of purified maltose binding protein (MBP), A-region-MBP, or P-region-MBP. Following blocking and washes as previously, purified A-region-MBP, P-region-MBP, and MBP were serially diluted two-fold in PBS-Tw and added to the wells, beginning at 500 ng/well. The plates were incubated overnight at 4°C and washed four times with PBS-Tw. MAbs 4-10A, 5-5D, and 6-11A were added to the wells at a 1:1000 dilution. Binding of the MAbs was traced with peroxidase-labeled goat anti-mouse IgG at a

1:2000 dilution, the plates were developed, and the absorbance was measured as previously described.

PCR and Construction of *S. mutans spaP* and *S. gordonii* M5 *sspA* and *sspB* Subclones

The P-regions of *S. gordonii* M5 *sspA* and *sspB* and several regions of *spaP* were amplified by PCR and cloned into the pGEX-4T-2 vector (Amersham Biosciences) for expression as fusion polypeptides with glutathione S-transferase (GST). Forward and reverse primers were designed based upon the published sequences of *sspA* and *sspB* (accession numbers U40025 and U40026 respectively) and the unpublished sequence of NG8 *spaP*. The primer sequences and engineered restriction sites are shown in Table 3. The following primers were used in these amplifications: *sspA* P-region (a.a. 808-1008) - TS41 and TS42, *sspB* P-region (a.a. 749-942) - TS42 and TS43, *spaP* A-region (a.a. 179-466) - TS24 and TS25, and *spaP* P-region (a.a. 816-1016) - TS28 and TS29. These primers were engineered with *Bam*HI and *Sal*I restriction sites to enable subsequent cloning into the pGEX-4T-2 expression vector.

PCR was performed for 30 cycles under the following conditions: denaturation at 95°C for 3 min; primer annealing at 51°C for 30 sec; and primer extension at 72°C for 40 sec. Final primer extension was carried out for an additional 7 min after the last cycle. The amplified PCR products of correct predicted size were cloned into the T/A cloning vector pCR2.1-TOPO (Invitrogen). This vector is supplied linearized with overlapping thymidine residues that can be ligated to the overhanging adenosine residues generated in the PCR-amplified products. Insertion of foreign DNA into this region prevents the expression of *lacZα* allowing for blue-white screening of *E. coli* transformants. Ligated pCR2.1 and PCR amplified products were used to transform *E. coli* DH5α by calcium

chloride [121]. Clones were screened on LB agar supplemented with 50 μg/mL kanamycin and 0.75 μg/mL X-gal. White colonies were picked and tested for the presence of *sspA*, *sspB*, and *spaP* insert DNA after alkaline lysis. Plasmid DNA from each recombinant was restricted with *Bam*HI (Promega) and *Sal*I (Promega), and electrophoresed on 0.7% (wt/vol) agarose. The appropriate sized DNA fragments were excised from the gel and purified using a Qiagen gel extraction kit. The *sspA*, *sspB*, and *spaP* fragments were ligated into *Bam*HI (Promega) and *Sal*I (Promega) linearized pGEX-4T-2 resulting in directional cloning downstream of the *gst* gene, which encodes the glutathione S-transferase (GST) of *Schistosoma japonicumi*. Ligated DNA was used to transform *E. coli* BL21 and transformants were selected for on LB agar supplemented with 100 μg/mL of ampicillin and 75 μg/mL X-gal. White colonies were screened for the presence of *spaP*-containing insert DNA as described previously.

The pGEX-4T-2-derived plasmids encoding the P1 A- and P-regions were designated pGEX-A and pGEX-P, respectively. The plasmids encoding the SspA and SspB P-region-GST fusions were designated pGEX-AP and pGEX-BP.

Purification and Confirmation of GST-Fusion Proteins

Recombinant *E. coli* harboring plasmids encoding GST-fusion proteins and pGEX-4T-2 as a control were grown for 16 hours in LB broth supplemented with 100 μg/ml of ampicillin (LB/A100) and passaged 1:100 into LB/A100. Following shaking at 25°C until an optical density at 600 nm of 0.5 was achieved, cultures were supplemented with 0.1 mM IPTG and grown for an additional 4 hours at 25°C.

To confirm expression of each fusion protein, lysates from each recombinant *E. coli*, as well as uninduced controls, were analyzed by SDS-PAGE and Western

immunoblot on 7.5% (wt/vol) polyacrylamide gels. Proteins in the gels were electroblotted onto nitrocellulose membranes for 1 hour at 100 volts. Replicate filters were stained with colloidal gold (Diversified Biotech, Boston, MA) or blocked for 1 hour at room temperature with PBS-Tw. Membranes were incubated overnight at room temperature with anti-GST rabbit polyclonal antisera (Amersham Biosciences) diluted 1:1000 in PBS-Tw. Membranes were washed four times with PBS-Tw prior to incubation for 2 hours at room temperature with affinity-purified peroxidase-labeled goat anti-rabbit conjugate (ICN/Cappell ICN Biomedicals, Aurora, OH) diluted 1:1000 in PBS-Tw. Membranes were washed twice with PBS-Tw and twice with PBS prior to development with 4-chloro-1-napthol solution for 30 min.

The P1-GST fusion proteins were purified by affinity chromatography using glutathione sepharose 4B (Amersham Biosciences). IPTG induced recombinant *E. coli* were resuspended in PBS containing 1 mM PMSF (phenylmethylsulfonyl fluoride) and lysed by sonication on ice 5 times for 15 seconds at power setting 3 using a Sonic 300 Dismembrator (ARTEK Systems Corporation, Farmingdale, NY). Triton X-100 was added to the sonicate to a final volume of 1% (vol/vol). Following a 30 minute incubation at 25°C, the sonicate was centrifuged for 10 minutes at 12,000 xg, and the supernatant was applied to the glutathione sepharose 4B. After repeated washes with PBS, the bound fusion proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8. The recovery of purified P1-GST fusion proteins was confirmed by Western immunoblot using anti-GST rabbit polyclonal antisera (Amersham Biosciences). Purified fusion proteins were quantified using the bicinchoninic acid (BCA) protein assay kit (Sigma) with bovine serum albumin as the standard.

Competitive Inhibition ELISA to Detect A-Region and P-Region Interaction

Individual wells of Costar High Binding plates (Corning Incorporated, Corning, N.Y.) were coated overnight at 4°C, in triplicate, with 100 µl of 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing 0.02% (wt/vol) sodium azide and 100 ng of P1. Coating buffer and unbound antigens were removed from the ELISA plate wells and unreacted sites were blocked with PBS-Tw and overnight incubation at 4°C. Plates were washed four times with PBS-Tw. Purified A-region-GST, P-region-GST, a 1:1 molar ratio of A-region-GST and P-region-GST, and GST were added to MAb 4-10A, diluted 1:8000, to a final concentration of 1 nM. Controls included MAb 4-10A alone and no primary antibody. The mixtures of P1-GST fusions and MAb 4-10A were incubated at 4°C for 30 minutes and then applied to the P1 coated ELISA plate at 100 µl per well. The plates were incubated for 2 hours at 37°C and washed four times with PBS-Tw. The binding of MAb 4-10A to the immobilized P1 was traced with peroxidase-labeled goat anti-mouse IgG (Cappell) at a 1:2000 dilution. The plates were developed with 4-CN, and absorbance was measured as previously described. The percent inhibition of MAb 4-10A binding to captured P1 was calculated as 100 − [(mean OD of Mab 4-10A + P1-GST fusion /mean OD of MAb 4-10A alone) × 100].

Binding Stoichiometry of the A- and P-Regions by Continuous Variation

A variation on the Job Plot [122] was used to measure the binding stoichiometry of the A- and P-regions required for the formation of the MAb 4-10A epitope. Purified A-region-GST and P-region-GST fusion proteins were diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing 0.02% (wt/vol) sodium azide and then mixed in 7 different molar ratios (0:6, 1:5, 2:4, 3:3, 4:2, 5:1, and 6:0) while maintaining a constant

total concentration of 0.67 μM. The mixtures were incubated at 4°C for 1 hour, and then 100 μl per well was applied to a Costar High Binding plate (Corning Incorporated, Corning, N.Y.) in triplicate. The plate was incubated overnight at 4°C. Coating buffer and unbound antigens were removed from the ELISA plate wells, and unreacted sites were blocked with an overnight incubation in PBS-Tw at 4°C. After washing 4 times with PBS-Tw, MAb 4-10A was added to the wells at a 1:1000 dilution. Wells were washed with PBS-Tw and peroxidase-labeled goat anti-mouse IgG was added to the wells at a 1:2000 dilution. After washing, the plate was developed with OPD, and absorbance was measured as above.

Analysis of P1 Translocation and the Contribution of the Alanine- and Proline-Rich Regions

Introduction of *spa*ΔA into *S. mutans* PC3370

The *spaP*ΔA DNA, including the promoter, was restricted by *Kpn* I from pTS20 and isolated by gel electrophoresis and purification with a Qiagen gel extraction kit. The purified *spaP*ΔA was ligated into the *Kpn* I site of the *E. coli – S. mutans* shuttle vector pDL289, creating pTS21, and introduced into *E. coli* DH5α by electroporation. Clones were screened on LB agar supplemented with 50 μg/mL ampicillin. Colonies were picked and tested for the presence of *spaP*ΔA insert DNA after alkaline lysis. pTS21 was subsequently introduced to the *S. mutans spaP*-negative mutant PC3370 by natural transformation. An overnight culture of PC3370 grown in THYE media was diluted 1:20 into THYE media containing 5% (vol/vol) sterile horse serum (THYHS). The culture was grown to Klett 100 at 37°C at which time pTS21 (μg/ml) was added. After an additional 30 minutes at 37°C, an equal volume of THYHS was added to the cultures.

Following 90 minutes at 37°C, transformants were screened on THYE agar supplemented with 500 μg/mL kanamycin. The sequences of all recombinant constructs were confirmed by the DNA sequencing core (University of Florida).

Analysis of P1ΔA Cell Surface Expression in PC3370

The *spaP* isogenic mutant PC3370 harboring plasmids encoding P1 (pMAD), P1ΔA (pTS21), P1ΔP (pMAJJ8) and vector only (pDL289) were grown for 16 hours at 37°C, the cells were harvested by centrifugation and washed twice with PBS. Cells were resuspended in PBS, and the densities of the suspensions were equalized at Klett 160. Twofold serial dilutions of the cell suspensions were made in PBS, and 100 μl of each dilution was applied in duplicate to two nitrocellulose membranes (Schleicher and Schuell) by using a 96-well dot blot manifold (Schleicher and Schuell). Wells were washed twice with 200 ml of PBS, and the filters were removed from the apparatus and blocked with PBS containing 0.25% (wt/vol) gelatin and 0.25% (vol/vol) Tween 20. Cell surface P1 was detected with rabbit antiserum 230 [37] or Mab 3-10E [119] as the primary antibodies diluted 1:500, peroxidase-conjugated goat anti-rabbit lgG and goat anti-mouse IgG as the secondary antibodies diluted 1:1000, and development with 4-chloro-1-naphthol solution.

RNA and Dot Blotting for Confirmation of P1ΔA Expression in PC3370

Following the manufacturer's protocol, RNA was isolated from stationary phase cultures of PC3370 harboring pDL289, pTS21 (P1ΔA), and pMAD (P1) using the Qiagen Rneasy kit (Qiagen, Valencia, CA). Total RNA concentration was measured by $OD_{260/280}$ nm and standardized to ~92 μg/ml by the addition of RNA dilution buffer (6x SSC, 20% formaldehyde). Samples were serially diluted two-fold, and 50 μl of each were applied

to a nylon membrane using a 96-well dot blot manifold (Schleicher & Schuell). The membrane was baked for 30 minutes at 120°C and incubated in DIG Easy Hyb (Roche, Indianapolis, IN) for 2 hours at 37°C. The membrane was probed overnight at 37°C with digoxigenin-labeled, PCR amplified, DNA complementary to the 3' end of *spaP*, nucleotides 3985-4125. The membrane was washed, blocked for 1 hour at 25°C in Roche blocking buffer, and incubated in alkaline phosphatase-labeled anti-digoxigenin antibodies. After washing in detection buffer, chemiluminence substrate, CSPD, was added and the membrane was exposed to Super Rx x-ray film (Fuji, Tokyo).

Western Immunoblot Analysis of Periplasm Extracts from *E. coli* MC4100 and CK1953 Harboring pUC18, pDC20, pDC9, and pTS20

Periplasm contents of *E. coli* DH5α harboring pUC18, pDC20, pDC9, or pTS20 and *E. coli* MC4100 and CK1953, a *secB* mutant [109], harboring pUC18 or pDC20 were extracted by osmotic shock [108]. Briefly, cells were grown for 16 hours at 37°C. The medium was supplemented with 0.3 mM IPTG to induce β-galactasidase expression, and the culture was incubated for an additional 2 hours at 37°C , harvested by centrifugation at 7000 x g for 10 minutes, and resuspended in 30 mM Tric-HCl/20% (wt/vol) sucrose, pH8.0, and EDTA to a final concentration of 1mM. The cells were incubated at 25°C for 10 minutes while shaking, harvested by centrifugation for 10 minutes at 10,000 x g, and resuspended in ice-cold 5 mM $MgSO_4$. Cells were next incubated in an ice bath for 10 minutes, centrifuged for 10 minutes at 10,000 x g, and again decanted. One molar Tris-HCl, pH 7.4, was added to the supernatant to a final concentration of 20 mM. The supernatant containing the periplasm contents was diluted 5:1 with SDS sample buffer and incubated for 5 minutes at 100°C. The cell pellets were resuspended in SDS-sample buffer and also heated for 5 minutes at 100°C. Proteins were separated on 7.5% (wt/vol)

SDS-polyacrylamide gels and transferred to nitrocellulose for 1 hour at 100V. Immunoblots were blocked and developed as described above for the dot blot assay.

Construction of a Bicistronic *spaP* for Expression of a Discontinuous P1.

The following engineering produced a genetic construct encoding *spaP* that expressed P1 as two independent fragments, the N-terminal 465 residues and the C-terminal 1095 residues. Fragments of *spaP* both upstream and downstream of the 3' end of the A-region were amplified by PCR and subsequently ligated together to create a "split" *spaP* (Fig. 3-5). Forward primer TS9k and reverse primer TS17 were used to amplify *spaP* DNA upstream of the 3' end of the A-region, including the *spaP* promoter. Forward primer TS18 and reverse primer TS10k were used to amplify *spaP* downstream of the A-region. Primers TS9 and TS10 were engineered with *Kpn*I restriction sites and primers TS17 and TS18 contain engineered *Xho*I restriction sites (Table 3). Primer TS17 also encodes multiple stop codons for the termination of the N-terminal P1 fragment translation. Primer TS18 contains the *spaP* ribosome binding site and encodes a start codon for translation of the C-terminal P1 fragment. Reactions were carried out in a UNO thermoblock thermocycler (Biometra, Tampa, FL) with plasmid-encoded *spaP*, pDC20 [54] as the template and HiFi DNA polymerase (Invitrogen) for 30 cycles under the following conditions: denaturation at 94°C for 30 seconds, primer annealing at 51°C for 1 minute, primer extension at 68°C for 2 minutes and 30 seconds or 72°C for 1 minute and 30 seconds; and a final extension at 72°C or 68°C for 7 min. The resulting 1,653- and 3,536-bp gene fragments were restricted with *Xho*I before being ligated together. The ligated fragments were gel purified and amplified by PCR as before using primers TS9 and TS10 under the following conditions for 30 cycles: denaturation at 94°C for 30 seconds, primer annealing at 58°C for 1 minute, primer extension at 68°C for 3

minutes 45 seconds; and 68°C for an additional 7 min. The PCR product was cloned into the pCR 2.1-TOPO vector, creating pTS30, which was introduced into E. coli Top 10 cells according to manufacturer's instructions. Clones were screened on LB agar supplemented with 50 μg/mL kanamycin and 0.75 μg/mL X-gal. White colonies were picked and tested for the presence of *spaP* insert DNA after alkaline lysis. Plasmid pTS30 from the recombinant was restricted with *Kpn*I and electrophoresed on 0.7% (wt/vol) agarose. The appropriate sized split *spaP* DNA fragment was excised from the gel and purified with the Qiagen gel extraction kit. The split *spaP* sequence was ligated into the *Kpn*I site of the shuttle vector pDL289, creating pTS31, and used to transform *E. coli* DH5α by electroporation. Transformants were selected for on LB agar supplemented with 50 μg/mL of kanamycin and 0.75 μg/mL X-gal. White colonies were screened for the presence of *spaP*-containing insert DNA as before. Sequences of all recombinant constructs were confirmed by the DNA sequencing core (University of Florida).

Evaluation of P1 Fragment Expression by Western Immunoblot.

E. coli DH5α harboring pTS30 was grown for 16 hours at 37°C, harvested by centrifugation, and lysed by boiling for 5 minutes in SDS-sample buffer (4% [wt/vol] sodium dodecyl sulfate [SDS], 2% [vol/vol] 2-mercaptoethanol, 20% [vol/vol] glycerol, 125 mM Tris-HCl [pH 6.8], 0.1 mg of bromophenol blue per ml). Proteins were separated by SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide preparatory gels by the method of Laemmli [117]. Proteins were electroblotted onto nitrocellulose membrane (Schleichter and Schuell, Keene, N.H.) for 1 h at 100 V by the method of Towbin et al. [118]. Immunoblots were blocked with PBS-Tw. Membranes were

incubated with A-region specific mAb 3-8D, A- and P-region dependent mAb 4-10A, and C-terminal specific mAb 5-3E [119, 120] at dilutions of 1:000. After washing, membranes were incubated in peroxidase-labeled goat anti-mouse IgG (Cappel) and developed with 4-chloro-1-naphthol solution.

Evaluation of Surface Expression of Discontinuous P1 in *S. mutans*

Plasmid pTS31 was introduced into *S. mutans* PC3370 by natural transformation as before. *S. mutans* NG8 and PC3370 harboring pDL289 and derivatives expressing P1 (pMAD) and discontinuous P1 fragments (pTS31) were grown for 16 hours at 37°C . Cells were harvested, applied to a nitrocellulose membrane, and surface expression of P1 was traced as before with MAbs, 3-8D, 4-9D, 4-10A, 5-5D, 6-11A, and 3-10E.

Introduction of *S. gordonii* SspA and SspB A-Regions into P1ΔA

DNA encoding the A-regions of *sspA* and *sspB* were amplified by PCR and ligated into the plasmid encoding P1ΔA, pTS21. pTS21 was constructed with two silent mutations that created a unique *Sfo* I recognition sequence at the site of the deleted A-region DNA [115]. PCR was used to amplify DNA fragments of *sspA* and *sspB* that encode 287 residues, which are homologous to the deleted A-region in the *spaP* construct P1ΔA. Primers TS19 and TS20 were used to amplify the *sspA* DNA fragment and primers TS21 and TS22 were used to amplify the *sspB* fragment (Table 3). Reactions were carried out in a UNO thermoblock thermocycler (Biometra, Tampa, FL) with chromosomal *sspA* and *sspB* as the templates and VENT polymerase (NEB) under the following conditions: (i) 94°C for 2 minutes; (ii) 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 and (iii) 72°C for an additional 7 min. The resulting 861 bp gene fragments were cloned into the *Sfo* I site of pTS21 with *E. coli* DH5α as the host strain. Plasmid DNA was isolated from clones and insert orientation was confirmed by

restriction digest and sequencing. The resulting plasmids, designated pTS22 (*sspA* A-region) and pTS23 (*sspB* A-region), were introduced into the *S. mutans spaP*-negative mutant strain PC3370 by natural transformation as previously described. Transformants were selected for their ability to grow on THYE containing 500μg/ml of kanamycin.

Western Immunoblot Analysis of Chimeric P1 Containing the A-region of *S. gordonii* SspA and SspB

Whole cell lysates of *E. coli*DH5α harboring pTS22 and pTS23 and mechanically lysed *S. mutans* PC3370 harboring the same plasmids were electrophoresed on 7.5% (wt/vol) SDS-PA gels, transferred to nitrocellulose, and traced with a panel of eleven anti-P1 mAbs as previously described.

Surface Expression of SspA and SspB in *S. mutans* PC3370

S. gordonii M5 SspA and SspB were expressed in the *spaP*-negative mutant PC3370, and translocation to the cell surface was determined by whole cell dot blot. Plasmids containing s*spA* driven by the *sspB* promoter, pGEM-*sspA* (unpublished), and *sspB*, pEB-5 [25] were kindly donated by D. Demuth (University of Pennsylvania, Philadelphia, PA). pGEM-s*spA* was linearized with *Sac*I and blunted with Klenow fragment, followed by a second digestion with *Sph*I. The *sspA* fragment was gel purified, ligated into the *Sph*I-*Sma*I site of the streptococcal shuttle vector pDL289 and introduced into PC3370 by natural transformation. pEB-5 [25] was digested with *Bam*HI and *Eco*RI and the appropriate-sized *sspB* band was gel purified. The *sspB* fragment was ligated into pDL289 and introduced into PC3370 as previously described. Transformants were selected for their ability to grow on THYE containing 500μg/ml of kanamycin.

Involvement of RopA (Trigger Factor) and DnaK in the Maturation and Translocation of P1

Evaluation of P1 Surface Expression by Whole Cell Dot Blot in the *S. mutans ropA* Mutant, TW90

Whole cell dot blots of TW90 [112], courtesy of Tom Wen (University of Florida, Gainesville, FL), were used to determine whether the reduction in adherence was due to a reduced level of surface localized P1. UA159 and TW90 were grown for 16 hours at 37°C in THYE broth. The cells were passaged into triplicate cultures at 1:50 in THYE broth, grown at 37°C to a Klett reading of 50, and passaged again in THYE broth at 1:50. Cells were grown to Klett readings of 20 and 150, harvested by centrifugation, and washed twice with PBS. Cells were resuspended in 50% of the original culture volume of PBS. Two-fold serial dilutions of the cell suspensions were made in PBS, and 100 µl of each dilution was applied to replicate nitrocellulose membranes (Schleicher and Schuell) by using a 96-well dot blot manifold (Schleicher and Schuell). Wells were washed twice with 200 µl of PBS, and the filters were removed from the apparatus and blocked with PBS containing 0.25% (wt/vol) gelatin and 0.25% (vol/vol) Tween 20. P1 was detected with five anti-P1 monoclonal antibodies [33] that recognize cell surface P1 as well as with rabbit polyclonal serum as the primary antibodies, each diluted 1:500. Secondary antibodies were goat anti-mouse IgG or goat anti-rabbit Ig (MP Biomedicals, Irvine, Ca) diluted 1:1000. The membranes were developed with 4-chloro-1-naphthol solution. Quantification of P1 surface expression was performed by densitometry using a Fluorchem imager and software (Alpha Innotech, San Leandro, Ca).

Evaluation of P1 Surface Expression by Whole Cell Dot Blot in *S. mutans* SM12, a Low-Level Expresser of DnaK

To examine the contribution of DnaK to P1 surface expression, a whole cell dot blot experiment as above was performed using *S. mutans* SM12, which was engineered to express approximately 5% of the level of DnaK as the parent strain, UA159 (Lemos and Burne, in preparation, University of Florida, Gainesville, FL)). *S. mutans* UA159 and SM12 were grown and harvested at early-log and stationary phases, Klett readings of 20 and 150, and surface expression was determined as previously detailed.

Analysis of *dnaK* Message Levels by Quantitative Real-Time PCR

Real-Time PCR was utilized to evaluate the effects of expression of the A- and P-region deletion constructs on *dnaK* mRNA level. Cultures of PC3370 harboring pDL289 (shuttle vector), pMAJJ8 (P1ΔP-region), and pMAD (P1), and pTS21 (P1ΔA-region) were grown in triplicate to Klett 100 after multiple passages, as above. RNA from each culture was isolated according to supplier's instructions using the Qiagen RNeasy kit (Qiagen, Valencia, CA). The total RNA concentration was measured by $OD_{260/280}$ nm. cDNA of *dnaK* and 16S RNA was synthesized from 0.5 μg of RNA using primer dnaKAS and 16sRVS respectively, and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) for 10 minutes at 25°C, 50 minutes 42°C, and 15 minutes at 70°C.

Transcript levels were determined by using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Reactions were performed in a 25-μl volume using the manufacturer's protocols. The forward primers for *dnaK* and 16s RNA were DnaKS and 16SFWD. Amplification was performed under the following conditions: 30 seconds at 95°C, followed by 40 cycles of 10 seconds at 95°C and 45 seconds at 60°C. Melt curve data was collected with an additional 100 cycles of 10 seconds starting at 60°C and increasing

by 0.4°C after cycle 2 and 15 seconds at 72°C. DNA amplification and fluorescence detection was performed with the iCycler IQ real-time PCR detection system and accompanying software (Bio-Rad). A standard curve was plotted for the reaction with values obtained from the amplification of known quantities of DNA from *dnaK*. 16s RNA was used to normalize RNA abundance for all reactions. For each experiment, cDNA was amplified from RNA which was freshly isolated from each of three parallel cultures of each transformant. Real-Time PCT was conducted on each cDNA sample triplicate resulting in 36 data points for each transformant

CHAPTER 3
RESULTS

Expression of Recombinant P1ΔA and Recognition by Anti-P1 Monoclonal Antibodies

Regions of P1 that contributed to the epitopes for eleven anti-P1 mAbs were identified based on immunoblot analysis of full-length and truncated P1 polypeptides. Several of these mAbs, 6-11A, 5-5D, 3-10E, and 1-6F, were initially mapped to the central region of the protein, which contained the P-region [120]. Deletion of the P-region (a.a. 826-996) from P1 eliminated the binding of mAbs 6-11A, 5-5D, 3-10E, and 4-10A. A region contributing to the binding of mAb 4-10A had also been mapped to the region just amino-terminal to the central region. Surprisingly, none of the P-region-dependent mAbs bound to a P-region subclone (a.a. 819-1017) [54] suggesting that the epitopes were complex and possibly conformational. Characterization of the epitope for mAb 6-11A was conducted by Rhodin et al [123]. Construction of several P1 subclones and analysis by Western immunoblot revealed that residues 465-1561 were not sufficient for the binding of mAb 6-11A. These data suggested that the P-region and residues amino-terminal of a.a. 465 were necessary for mAb 6-11A binding. Taken together with crystal structure data indicating that the A-region and P-region may be in close proximity [30], we elected to examine whether the A-region contributed to a complex structure also involving the P-region. A *spaP* gene lacking DNA encoding the A-region (a.a. 179-466) was constructed by PCR and cloned into pUC18, creating pTS20, as detailed in Chapter 2. P1 lacking the A-region (P1ΔA) was detectable by Western immunoblot in whole cell

lysates of recombinant *E. coli* DH5α using anti-P1 polyclonal antibodies (data not shown). While full-length P1 migrates approximately 20-kD larger than its predicted ~165 kD on SDS-polyacrylamide gels, P1ΔA, like P1ΔP, migrates at its predicted molecular weight. The effect of deleting the A-region on the antigenicity of P1 was examined by Western blotting utilizing the eleven anti-P1 monoclonal antibodies [33]. Deletion of the A-region from P1 eliminated the reactivity of five of the eleven mAbs (Figure 2). Three of the non-reactive mAbs, 4-10A, 5-5D, and 6-11A, are also not reactive with P1ΔP [54]. Reactivity of mAbs 5-3E, 2-8G, 3-3B, and 6-8C, which are specific to the C-terminus of P1 [120], confirmed that the deletion of the DNA encoding the A-region did not disrupt the reading frame. The Western blot also shows that like P1ΔP, P1ΔA is stably expressed and easily detectable in *E. coli*.

Evaluation of P1ΔA Expression in *S. mutans*

When expressed in *S. mutans*, P1ΔP was unstable and not detected on the cell surface [54]. To determine whether the deletion of the A-region resulted in similar P1 characteristics, P1ΔA was expressed in the *spaP* mutant PC3370. Whole cell dot blot analysis of PC3370 harboring pDL289 (vector), pMAD (P1), or pTS21 (P1ΔA) was used to examine whether the A-region, like the P-region, is necessary for P1 surface expression in *S. mutans*. These results are shown in Figure 3. Two-fold serial dilutions of the cells were applied to the nitrocellulose membrane in duplicate. The positive control PC3370C expressing full-length P1 (row 4) demonstrated the reactivity of the antiserum with surface expressed P1. Negative controls, PC3370 and PC3370A, vector only, (rows 1 and 2) showed lack of reactivity of the antiserum with cells lacking P1. PC3370 harboring pTS21 encoding P1ΔA (row 3) was not reactive with the polyclonal

antiserum indicating a lack of surface expression of P1. These results indicated that P1ΔA was not translocated to the surface of PC3370. No P1ΔA was detected in spent culture liquor, although P1 was found in the spent culture liquor of PC3370C (complemented *spaP* mutant) and NG8 (wild-type) (data not shown).

To determine if the lack of detectable P1ΔA on the surface was due to a problem with translocation out of the cytoplasm, cell lysates were examined for detectable P1ΔA. NG8, PC3370A, PC3370C, PC3370, and PC3370 harboring pTS21 were subjected to mechanical lysis in a Mini Beadbeater apparatus, and samples were analyzed by Western blotting (data not shown). Full-length P1 was present in both cell extracts and cell debris of NG8 (wild-type) and PC3370C (complemented *spaP* mutant). P1ΔA was not detected in either the cell extract or the cell debris of PC3370 harboring pTS21, and no P1 was observed in the negative controls, PC3370A (vector only) or PC3370.

Evaluation of *spaP*-Specific mRNA in PC3370 Harboring the Deletion Construct pTS21

With a lack of detectable P1ΔA in PC3370, an RNA dot blot was used to confirm that *spaP*ΔA was transcribed from the pDL289 shuttle vector in PC3370 (Figure 4). Dilutions of total cellular RNA were probed with a digoxinin-labeled probe corresponding to the 3' end of *spaP*. The top two rows correspond to the negative controls, PC3370 and PC3370A harboring the vector only. The third row contains RNA from PC3370 harboring pTS21 and the bottom row contains RNA from the positive control, PC3370 harboring pMAD. The dot blot shows that *spaP*ΔA message is expressed at levels equivalent to the full-length *spa*P expressed from pMAD.

Evaluation of Secretion of P1, P1ΔA, and P1ΔP in *E. coli*

Since P1ΔA and P1ΔP were undetectable and possibly unstable in *S. mutans* while being clearly detectable in *E. coli*, the use of *E. coli* as a model to determine the secretion competency of these proteins was examined. To this end, periplasmic extracts of *E. coli* DH5α harboring pUC18, pDC20, pDC9, or pTS20 (vector only, and expressing P1, P1ΔP, and P1ΔA respectively) were prepared by osmotic shock, and the presence of P1 and derivatives was detected by electrophoresis on SDS-polyacrylamide gels followed by Western immunoblotting. These results are shown in Figure 5. Lanes marked "C" contain cellular lysates and lanes marked "P" contain periplasmic extracts. Lanes marked pDC20 contain cellular extracts from *E. coli* DH5α harboring pDC20 (full-length P1) and clearly show that P1 is present in both the cytoplasm and the periplasm. Lanes marked pDC9 show cellular fractions from DH5α harboring pDC9 and show that P1ΔP is present in the cytoplasm, but absent from the periplasm. Lanes pTS20 correspond to cell fractions from DH5α harboring pTS20 and show that, like P1ΔP, P1ΔA is present in the cytoplasm, but not translocated to the periplasm. Lanes marked pUC18 are cellular lysates and periplasm extracts from *E. coli* harboring pUC18 (vector only). These results suggest that *E. coli* may be a viable model for the study of the intramolecular requirements for P1 translocation.

Interaction of the A- and P-Regions Detected by ELISA

The demonstration that the binding of mAbs 4-10A, 5-5D, and 6-11A to P1 were dependent upon the simultaneous presence of the A- and P-regions and work by van Dolleweerd et al [124] characterizing a complex epitope comprised of the P-region and a fragment of P1 containing the A-region suggested a possible interaction between these

domains. To determine whether the isolated A- (a.a. 186-469) and P-regions (a.a. 819-1017) were capable of such an interaction, ELISA was used to evaluate binding (Figure 6). To facilitate protein purification, the A-region and P-region of P1 were expressed as fusions with maltose-binding protein (MBP), pMA41 and pMA3 respectively (Table 1). Purified P-region-MBP or MBP alone as a negative control was immobilized in ELISA plate wells. After washing and blocking the plates, two-fold serial dilutions of A-region-MBP were added to the wells. Binding of A-region-MBP to the immobilized proteins was detected by the A-region-specific MAb 3-8D. As shown, the A-region-MBP bound to P-region-MBP in a dose-dependent manner, but not to MBP alone. MBP alone did not bind to MBP or to P-region-MBP (data not shown).

Restoration of Epitopes by the Interaction of the A- and P-Regions

The requirement for the simultaneous presence of both the A- and P-regions for MAb 4-10A, 5-5D, and 6-11A binding to P1 suggested that both of these regions contribute to the epitopes for these mAbs. Reactivity of these mAbs against ELISA plates coated with the A- and P-regions was tested as above (Figure 7). The ELISA revealed that while the mAbs did not react to the A- or P-regions alone, they did react to the wells containing both regions. Additionally, MAb 4-10A reacted equally well regardless of which region is immobilized, while mAbs 5-5D and 6-11A clearly reacted better when the P-region is immobilized to the plate.

Inhibition of MAb 4-10A Binding to P1 by an A- and P-Region Complex

Competitive inhibition ELISA was used to assay the ability of the A- and P-regions to interact in solution phase. It was previously shown that the binding of MAb 4-10A to P1 was dependent upon the presence of both the A- and the P-regions. In this assay, the ability of A-region and P-region-GST fusions alone and together to inhibit MAb 4-10A

binding to immobilized P1 was analyzed. As shown in Figure 8 as a percentage of inhibition, MAb 4-10A binding to immobilized P1was not inhibited by A-region-GST, P-region-GST, or GST alone. However, MAb 4-10A binding was inhibited by a mixture of A-and P-region-GST as well as by P1 in solution.

Stoichiometry of the A- and P-Region Interaction

A quasi-continuous variation assay was performed to determine the stoichiometry of the A- and P-region interaction required for the reconstitution of the epitope required by MAb 4-10A. Varying molar ratios of A- and P-region-GST fusion proteins were mixed while maintaining a constant total molar concentration. The A-region/P-region mixtures were immobilized on a 96-well ELISA plate, and binding of MAb 4-10A to the mixtures was traced with peroxidase-labeled goat anti-mouse antibody. The maximum binding of MAb 4-10A to the A-region/P-region complex was clearly seen at a 1:1 molar ratio (Figure 9).

Interaction of P1, SspA, and SspB A- and P-Regions

The A- and P-regions of P1 are approximately 70% identical to the same regions in the *S. gordonii* M5 SspA and SspB, also members of the antigen I/II family. To determine whether the SspA and SspB A- and P-regions exhibited the same binding characteristics as the P1 regions, ELISA was used to analyze their ability to interact with one another and with the A- and P-regions of P1. A- and P-region-GST fusion proteins were purified by affinity column chromatography and immobilized to ELISA plates as previously described. Two-fold serial dilutions of A-region-GST fusion proteins were incubated with the immobilized P-region-GST proteins, and binding was traced with the A-region specific mAb 3-8D as before. Figure 11 shows that the A-regions of P1, SspA, and SspB are capable of binding to the P-regions of all three of the antigen I/II family

proteins. As measured by ELISA and Western blot (Figure 10) mAb 3-8D reacts equally to each of the A-region-GST fusions.

Anti-P1 mAb Epitope Restoration by the Interaction of A- and P-Regions of SspA and SspB

Based on the demonstrated restoration of epitopes by the interaction of the A- and P-regions of P1, the interactions of the A- and P-regions of SspA and SspB, and the reactivity of mAbs 4-10A and 5-5D to full-length SspA and SspB (Figure 12), restoration of epitopes for the mAbs by the interaction of the A- and P-regions of SspA and SspB was examined. Reactivity of mAbs 4-10A, 5-5D, and 6-11A with a combination of the A- and P-regions was tested by ELISA as previously described. As shown in Figures 13 through 18, none of the mAbs reacted to the A- or P-regions alone; however, the results showed that all three mAbs reacted to all A-region/P-region interactions in which a P1 fragment was the overlaid moiety. The binding of all three mAbs was also restored upon the interaction of any P-region to the immobilized A-region of P1. The interaction of SspA A-region with immobilized P1 P-region was also able to restore binding of mAb 5-5D. MAb 5-5D binding was also restored to a more limited extent when the P-region of P1 was overlaid on the immobilized A-region of SspA. In summary, all heterologous A- and P-regions interacted, yet not all of the interactions restored anti-P1 mAb epitopes.

Introduction of the A-Regions of SspA and SspB into P1ΔA

The identification of the interaction between the A-regions of SspA and SspB and the P-region of P1 including the restoration of the mAb 5-5D epitope suggested that some degree of P1 structure was attained but native structure was not fully restored at the polypeptide level. To determine whether introduction of the A-regions of SspA and SspB

into P1ΔA could restore native structure and translocation of the deletion construct, each A-region was ligated in-frame into the site of the deletion in P1ΔA. The homology between each of the A-regions is illustrated in Figure 19. The resulting chimeric P1 proteins were examined for restoration of mAb binding by Western immunoblot (Figure 20). The binding of mAb 3-8D demonstrated that the *S. gordonii* A-regions had been inserted into P1ΔA and were in-frame (panel A). Restoration of binding was only seen for mAb 5-5D with the chimeric P1 protein containing the A-region of SspA (panel B). Carboxy-terminal specific mAb 6-8C reacted to both chimeric proteins indicating that the insertion was in-frame and that the proteins were not truncated (data not shown). The bands that mAb 6-8C reacted to were of the same molecular weight as the band that 5-5D reacted to in panel B. Full-length P1 migrates on SDS-polyacrylamide gels near 185 kD. Oddly, neither chimeric protein appeared to migrate slower than P1ΔA. It is apparent that the introduction of the A-regions did not restore native P1 migration characteristics to the proteins.

Stability and Translocation of Chimeric P1 Containing the A-Regions of SspA and SspB.

Although full native structure based upon recognition by all A- and P-dependent mAbs was not achieved, the binding of 5-5D to the SspA chimeric construct indicated that some degree of A- and P-region interaction had been restored, therefore it was still of interest to examine the possible restoration of translocation of the chimeric molecules to the cell surface. To insure that there was not an intrinsic problem with the translocation of the *S. gordonii* proteins to the surface of *S. mutans*, SspA and SspB were expressed in PC3370. Whole cell dot blots were performed and expression was traced with mAb 5-5D as it demonstrated the strongest cross-reactivity with SspA and SspB in Western blots.

Figure 21 demonstrates that both SspA and SspB were translocated to the surface in *S. mutans*.

The surface expression of chimeric P1 containing the A-regions of SspA and SspB was also examined by whole cell dot blot. PC3370 harboring pDL289, pMAD (P1), pTS21 (P1ΔA), pTS22 (P1ΔA+SspA A-region), and pTS23 (P1ΔA+SspA A-region) were bound to nitrocellulose membrane using a 96-well dot blot manifold. Surface expression of P1 was traced with mAbs 4-10A and 5-5D as they are reactive to P1 on the cell surface and were reactive to SspA and SspB by Western immunoblot (Figure 22). No surface expression of either chimeric P1 was detected on the surface of PC3370. No full-length chimeric P1 proteins were detected in cell lysates of transformed PC3370 by Western blot with C-terminal specific anti-P1 mAbs (Figure 23, upper panel). Breakdown products of the proteins were, however, detected with the A-regions specific mAb 3-8D (Figure 23, lower panel).

Evaluation of the Involvement of SecB in the Secretion of P1, P1ΔA, and P1ΔP in *E. coli*

It is presumed that P1 is translocated to the cell surface via the general secretory pathway and the Sec translocase [86]. The two major routes to the Sec translocase are via the chaperones, signal recognition particle (SRP) or SecB. In *S. mutans*, however, P1 is secreted in the absence of the SRP pathway and *S. mutans* does not possess SecB or a known ortholog. To determine whether the translocation of P1 is dependent upon SecB in *E. coli* and possibly a SecB ortholog in *S. mutans*, secretion of P1 to the periplasm was examined in the *E. coli* SecB-negative mutant CK1953 [109]. Periplasmic extracts of CK1953 and the wild-type MC4100 expressing P1were prepared by osmotic shock, and the presence of P1 was detected by Western immunoblotting using mAbs 5-3E, 2-8G,

and 6-8C. These mAbs are specific to the carboxy-terminus of P1 and were used to insure that only full-length molecules were traced. These results are shown in Figure 24A. Lanes marked "C" contain cellular lysates and lanes marked "P" contain periplasm extracts. The host and plasmid expressed is indicated above each pair of lanes. The first pair of lanes contains cellular extracts from MC4100 harboring pDC20 (full-length P1) and show that P1 is detected in both the cytoplasm and the periplasm. The cellular fractions of the second pair of lanes contains cellular extracts from the SecB$^-$ mutant CK1953 harboring pDC20 and show that, like in MC4100, P1 is detected in both the cytoplasm and the periplasm. The final pair of lanes corresponds to cell fractions from MC4100 harboring pUC18 (vector only). The above cell extracts were also analyzed by Western immunoblotting for the cytoplasmic protein β-galactosidase to confirm the integrity of the periplasm extractions (Figure 24B). No β-galactosidase was detected in the periplasmic extracts. These results show that P1 translocation in *E. coli* is not dependent on SecB, the chaperone that is central to the general secretory pathway of *E. coli*.

Expression of Discontinuous P1 and Recognition by Anti-P1 Monoclonal Antibodies

It has been proposed that proteins frequently contain "uncleaved intramolecular chaperone-like fragments". These fragments are believed to assist in protein stabilization and folding by binding to adjacent regions [125]. Intramolecular chaperones (IMC) have been identified in a number of proteases, including a proline-rich IMC in the *Limulus* Factor C [79] in which deletion of the IMC resulted in a malfolded and non-secreted protein. Complementation of a secretion defect resulting from an IMC deletion has been demonstrated in the *Pseudomonas aeruginosa* elastase, LasB. McIver et al [126]

successfully rescued secretion and activity by expressing the IMC in *trans*. Additionally, not all secreted proteins are translocated in an unfolded conformation. The TAT system is capable of secreting proteins that are first folded in the cytoplasm [127]. Although the TAT system has not been found in *S. mutans*, it was of interest to determine whether the A- or P- regions possessed IMC activity and whether such an interaction would result in the translocation of a non-linear or folded P1. To examine this, a *spaP* gene engineered to express P1 as two peptides (a.a. 1-480 and a.a. 481-1561, see Figure 25) was constructed by PCR and cloned into pCR2.1, creating pTS30, as detailed in Methods and Materials.

The predicted molecular weights of the P1 fragments are 51 kD for the 480 residue N-terminal fragment and 119 kD for the 1081 residue C-terminal fragment. Cell lysates of *E. coli* harboring pTS30 were examined by Western blotting utilizing anti-P1 mAbs, 3-8D, 4-10A, and 5-3E (Figure 26). MAb 3-8D is specific to the A-region of P1 [35], reactivity of MAb 4-10A has been shown to be dependent upon the simultaneous presence of both the A- and P-regions, and MAb 5-3E is specific to the C-terminal end of P1 [120]. In lane A, MAb 3-8D is shown to be reactive with bands migrating between approximately 65 and 80 kD. In lane B, MAb 4-10A reacts with a band that corresponds to the molecular weight of the C-terminal fragment at 119 kD. The binding of mAb 4-10A suggests that enough N-terminal fragments co-migrate with the 119 kD C-terminal fragment to restore epitope recognition by this antibody. In lane 3, mAb 5-3E also reacts with a 119 kD band. The reactivity of all three mAbs indicates that both the N-terminal and C-terminal P1 fragments are expressed and detectable in *E. coli*.

Evaluation of Surface Expression of Discontinuous P1 in *S. mutans*

The *spaP* mutant PC3370 was used as the host for plasmids pMAD and pTS31, encoding full-length P1 and discontinuous P1 respectively. Whole cell dot blot analysis was used to examine whether an interaction of the A- and P-regions, when expressed in *trans*, could result in translocation of the fragments to the cell surface. These results are shown in Figure 27. The positive controls *S. mutans* NG8 (wild-type) and PC3370C expressing full-length P1 (columns A and C) demonstrate the reactivity of the mAbs with surface expressed P1. MAb 3-8D has previously been shown to be unreactive with full-length P1 on the cell surface. The negative control, PC3370A, harboring the vector only, (column B) showed lack of reactivity of the mAbs with cells lacking P1. PC3370 harboring pTS31 encoding the P1 fragments (column D) was not reactive with the mAbs indicating a lack of surface expression of the P1 fragments. These results indicated that P1 fragments were not anchored to the surface of PC3370. The N-terminal fragment was, however, detected in spent culture liquor with MAb 3-8D (data not shown).

To determine if the P1 fragments were detectable in *S. mutans* cell lysates, PC3370 harboring pTS31 was subjected to mechanical lysis in a Mini Beadbeater apparatus and samples were analyzed by Western immunoblotting (data not shown). While both the N- and C-terminal fragments were detected in *E. coli,* only the N-terminal fragment was present in the *S. mutans* cell extracts, indicating that the C-terminal fragment was unstable in *S. mutans*.

Surface Expression of P1 in a RopA-Deficient *S. mutans* and Bacterial Adherence to Salivary Agglutinin

The first chaperone encountered by nascent polypeptides is believed to be the polyprolyl isomerase (PPIase), RopA or trigger factor. Trigger factor is involved in

protein secretion and maturation. The involvement of RopA in the expression of functional P1 was analyzed using an adherence assay. P1 mediates binding to salivary agglutinin, and the binding can be inhibited by the P1-specific monoclonal antibody (mAb) 4-10A, but not by the P1-specific mAb 6-11A [33]. Adherence of *S. mutans* TW90, a RopA-deficient mutant [112], to human salivary agglutinin immobilized on an F1 sensor chip was assayed using the BIAcore 3000 (BIAcore AB, Uppsala, Sweden) by Monika Oli by the method described in [128]. Briefly, agglutinin was immobilized on the BIAcore F1 sensor chip surface, and suspensions of *S. mutans* UA159 (wild-type) and TW90 (Δ*ropA*) in adherence buffer were injected onto the sensor chip. A substantial reduction in adherence (> 50%) in three separate assays was observed for the RopA-deficient cells (Brady laboratory, unpublished). The complete inhibition of adherence of both UA159 and TW90 by the addition of anti-P1 mAb 4-10A indicated that the residual adherence was P1 mediated (Brady laboratory, unpublished).

In light of the laboratory's findings that the function of P1 appeared to be altered in a *ropA*-negative strain, whole cell dot blots of TW90 were used to determine whether the reduction in adherence was due to a reduced level of surface localized P1 in the *ropA*-negative background. Cells were grown to early-log and stationary phases, and samples were standardized for cell number by absorbance measurements. Replicate blots were reacted with mAbs 3-8D, 4-9D, 4-10A, 5-5D, 6-11A, 3-10E, 1-6F, 5-3E, 2-8G, 3-3B, or 6-8C. Quantification of P1 surface expression was performed by densitometry using a Fluorchem imager and software (Alpha Innotech, San Leandro, Ca). The mAb 4-10A results shown in Figure 28 are representative of all data. There were no differences detected in the surface expression of P1 between wild-type UA159 and TW90.

Analysis of P1 Surface Expression in an *S. mutans* Mutant Expressing Low-Levels of DnaK

The route of P1 translocation to the cell surface and the chaperones involved are unknown. P1 is secreted in the absence of the chaperones SRP, SecB, and RopA, although RopA appears to affect P1 function. DnaK binds to proline-rich proteins [129] and is involved in chaperoning a wide variety of proteins. DnaK also has a pool of substrates that overlaps with RopA [104]. The contribution of DnaK to P1 surface expression was examined by whole cell dot blot as was performed with the RopA mutant. The experiment was performed using *S. mutans* SM12, which was engineered to express approximately 5% of the level of DnaK as the parent strain, UA159 (Lemos and Burne, in preparation, University of Florida, Gainesville, FL). *S. mutans* UA159 and SM12 were grown and harvested at early-log and stationary phases, samples were standardized for cell number by absorbance measurements, and surface expression was determined as previously detailed. Figure 29 shows that there is a significant reduction in the amount of surface expressed P1 at early-log growth in SM12 ($P<0.0001$), but P1 levels were equal in both strains at stationary phase (data not shown).

Evaluation of *dnaK* mRNA Expression in *S. mutans* PC3370 Harboring pDL289, pMAJJ8, pMAD, and pTS21

The reduction of P1 surface expression seen in the early-log phase of SM12 suggested that DnaK might have a role in P1 translocation. Changes in *dnaK* mRNA levels in response to the expression of P1 and P1 deletion constructs in *S. mutans* could indicate an interaction between the chaperone and the P1 proteins. To this end, quantitative Real-Time PCR was utilized to measure levels of *dnaK* mRNA expression. DnaK message was quantified from total RNA isolated from early-log phase cultures of PC3370 harboring pDL289 (shuttle vector), pMAJJ8 (P1ΔP-region), and pMAD (P1),

and pTS21 (P1ΔA-region). Compared to the vector only control or PC3370 complemented with full-length P1, the level of *dnaK* message was significantly decreased ($P<0.005$ and $P<0.05$, respectively) in the presence of P1ΔP and increased ($P<0.005$ and $P<0.05$, respectively)in the presence of P1ΔA (Figure. 30) The mRNA levels of 16S RNA were used as an internal control, and no significant difference was found between samples ($P<0.38$).

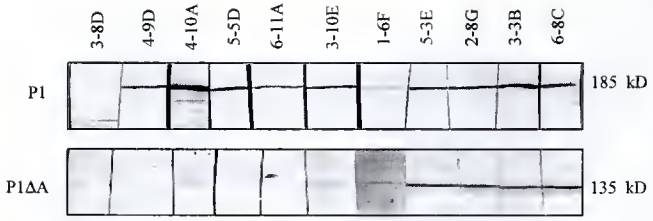

Figure 2. Western blot analysis of P1 and recombinant P1 lacking the A-region (P1ΔA). The reactivity of eleven anti-P1 mAbs against whole cell lysates of *E. coli* harboring pDC20 (P1) or pTS20 (P1ΔA) were analyzed by Western blot to determine the effect of the A-region deletion on antigenicity. The mAbs used are listed above each strip. The epitope of mAb 3-8D is within the A-region, however mAb 3-8D does not bind to full-length P1. The reactivity of mAbs 4-10A, 5-5D, 6-11A, and 3-10E are dependent upon the P-region. The reactivity of mAbs 5-3E, 2-8G, 3-3B, and 6-8C are dependent upon the C-terminal terminal third of P1.

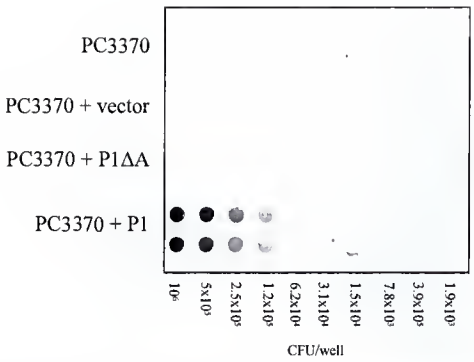

Figure 3. Lack of surface expression of P1 devoid of the A-region. Whole cell dot blots of S. mutans *spaP* isogenic mutant PC3370, PC3370 harboring shuttle vector pDL289 alone, and PC3370 harboring the pDL289 construct encoding P1ΔA or full-length P1. Blots were reacted with anti-P1 mAbs 1-6F and 3-10E. These antibodies had been shown in previous experiments to react with recombinant P1ΔA. Identical results were obtained using a polyclonal anti-P1 rabbit antiserum (data not shown).

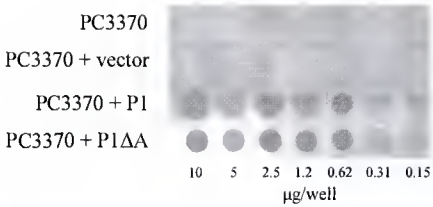

Figure 4. RNA dot blot analysis of *spaP*-specific mRNA levels in the *S. mutans spaP*-negative mutant PC3370 and derivatives. Twofold serial dilutions of total cellular RNA, beginning with 10 mg, were probed with DNA encoding the C-terminus of *spaP*. From top to bottom, the rows contain mRNA from PC3370, PC3370A (vector only), PC3370C (full-length *spaP*), and PC3370 harboring pTS21 (*spaP* with A-region encoding DNA deleted), respectively.

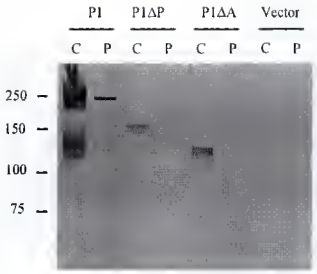

Figure 5. Western immunoblot of cytoplasm (C) and periplasm (P) fractions of *E. coli* DH5α harboring pUC18 derived plasmids expressing full-length P1 (pDC20), P1ΔP (pDC9), P1ΔA (pTS20), and vector alone detected with C-terminus specific mAbs 5-3E, 6-8C, and 2-8G. Migration of molecular weight standards are indicated in kilodaltons.

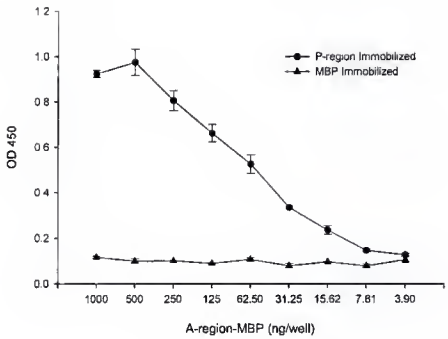

Figure 6. Demonstration of A-region and P-region interaction by ELISA. 100 ng of P-region-maltose binding protein (MBP) fusion polypeptide (·) or MBP alone (Δ) were used to coat ELISA plate wells. Two-fold serial dilutions of purified A-region-MBP fusion polypeptide starting at 1000 ng/well were added to the coated wells, and binding of the A-region to the P-region or to the MBP negative control was detected with the A-region specific MAb 3-8D.

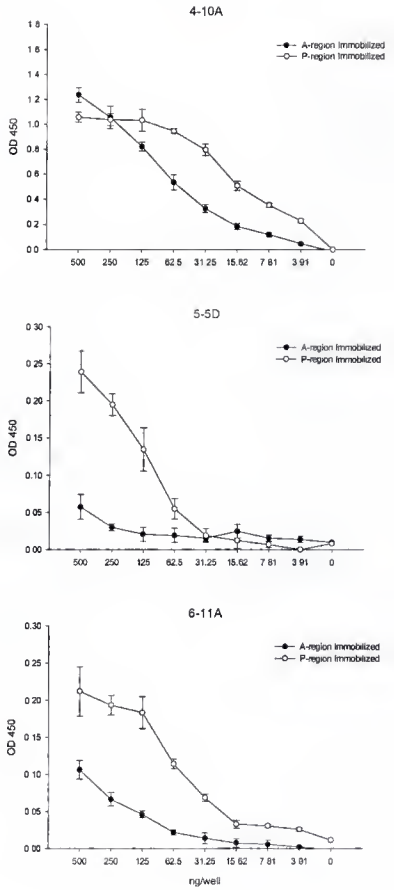

Figure 7. Restoration of epitopes by A- and P-region interactions as measured by ELISA. 500 ng of P-region-MBP fusion polypeptide, A-region-MBP, or MBP were used to coat ELISA plate wells. Two-fold serial dilutions of purified A-region-MBP starting at 500 ng/well were added to the P-region and MBP coated wells and vice versa. MAbs 4-10A, 5-5D, and 6-11A were tested for reactivity. Panel titles indicate the mAb tested and the legends indicate the P1 fragment that was immobilized. A- and P-regions did not interact with MBP alone and no mAb binding was detected with the controls (data not shown).

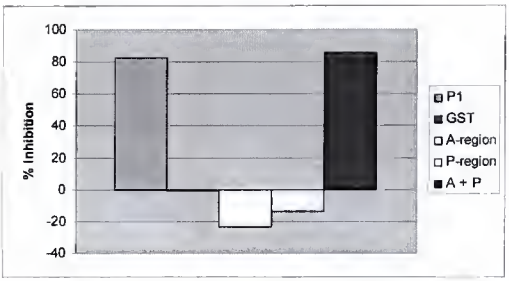

Figure 8. Inhibition of anti-P1 MAb 4-10A binding to immobilized P1 in ELISA. To determine whether the A- and P-region polypeptides could interact in solution and produce an epitope recognized by mAb 4-10A, the antibody was mixed with soluble P1, glutathione S-transferase (GST), A-region-GST, P-region-GST, or a 1:1 molar mixture of A-region-GST and P-region-GST polypeptides. The mAb 4-10A mixtures were applied to P1 immobilized to an ELISA plate, and inhibition of binding to the immobilized P1 was measured. Bars indicate percent inhibition.

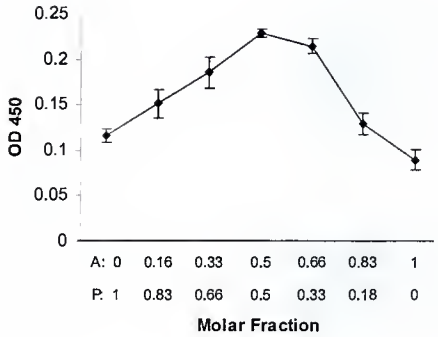

Figure 9. Stoichiometry of the mAb 4-10A epitope. Varying molar ratios of P1 A- and P-region polypeptides with a constant total concentration of 3.3 pmoles were immobilized in ELISA plate wells and epitope restoration was detected with mAb 4-10A. The experiment was performed in triplicate and standard deviation is represented by the error bars.

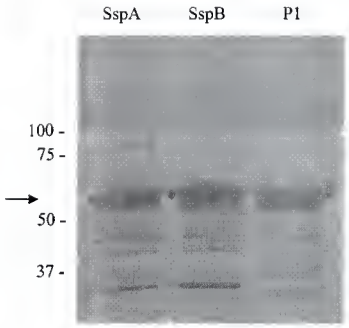

Figure 10. Demonstration of similar level of mAb 3-8D reactivity to A-region-GST fusion polypeptides of SspA, SspB, and P1 by Western immunoblot.

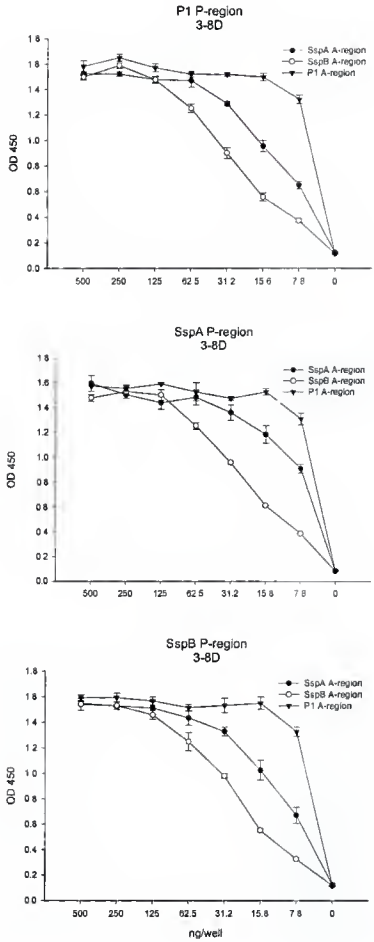

Figure 11. Demonstration of interactions between the A- and P-regions of different antigen I/II proteins. Panel titles indicate the source of the immobilized P-regions. Binding of the different A-regions identified in the legends were traced with the cross-reactive A-region specific mAb 3-8D. Legends identify the overlaid polypeptides.

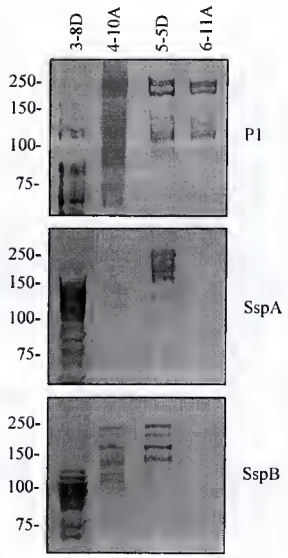

Figure 12. Evaluation of reactivity of A- and P-region dependent anti-P1 mAbs with P1, SspA, and SspB. Whole cell lysates of *E. coli* DH5α harboring pDC20 (P1), pDDA (SspA), and pEB-5 (SspB) were electrophoresed on 7.5% SDS polyacrylamide gels, transferred to nitrocellulose and probed with the anti-P1 mAbs shown above. The indicated molecular weights are in kilodaltons.

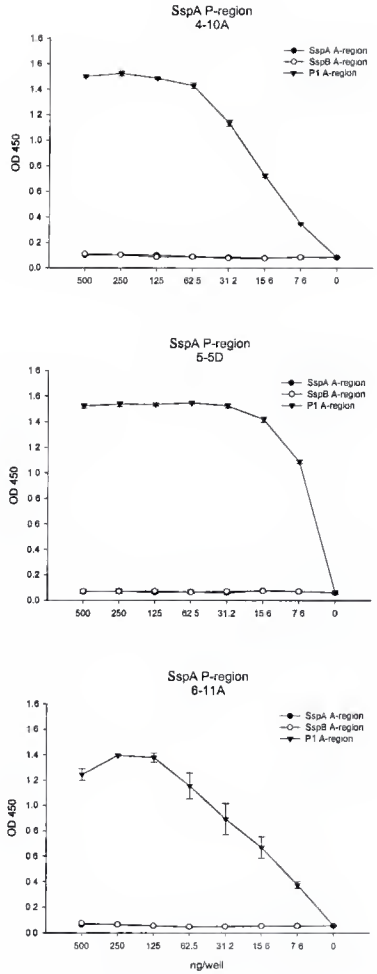

Figure 13. Restoration of epitopes by the interaction of various antigen I/II A-regions with the immobilized P-region of SspA. Panel titles indicate the source of immobilized P-region and the mAb tested. Legend indicates the source of the overlaid A-regions.

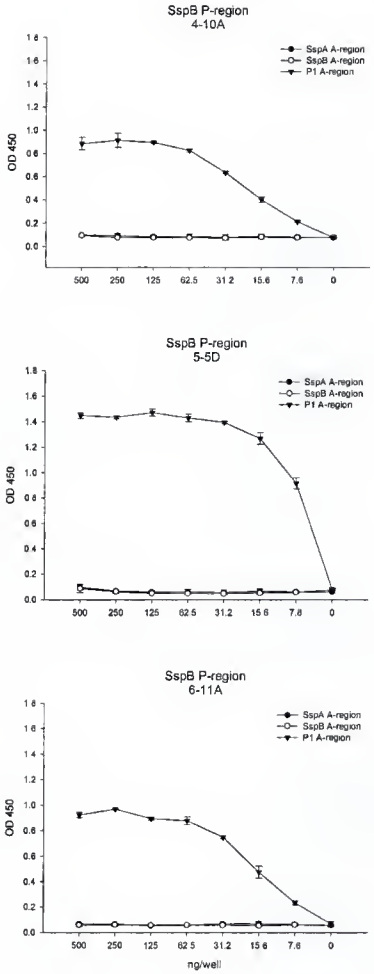

Figure 14. Restoration of epitopes by the interaction of various antigen I/II A-regions with the immobilized P-region of SspA. Panel titles indicate the source of immobilized P-region and the mAb tested. Legend indicates the source of the overlaid A-regions.

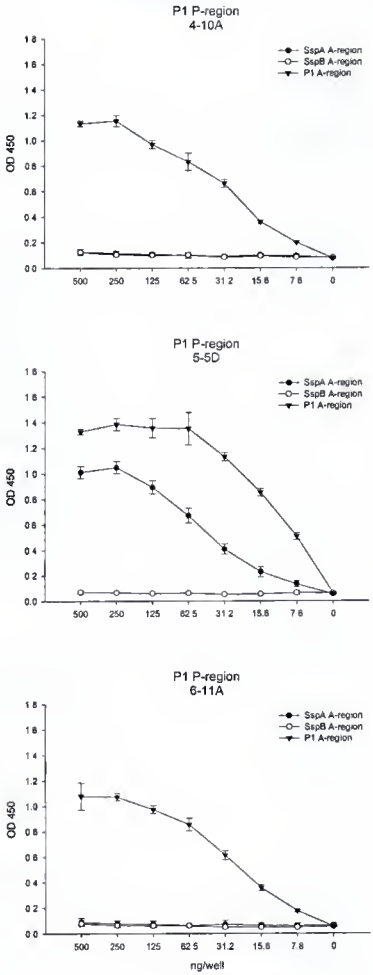

Figure 15. Restoration of epitopes by the interaction of various antigen I/II A-regions with the immobilized P-region of SspA. Panel titles indicate the source of immobilized P-region and the mAb tested. Legend indicates the source of the overlaid A-regions

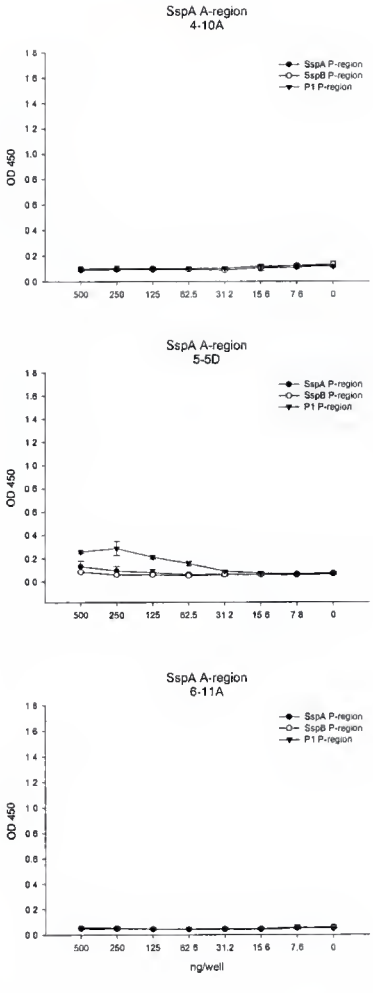

Figure 16. Restoration of epitopes by the interaction of various antigen I/II A-regions with the immobilized A-region of SspA. Panel titles indicate the source of immobilized P-region and the mAb tested. Legend indicates the source of the overlaid P-regions

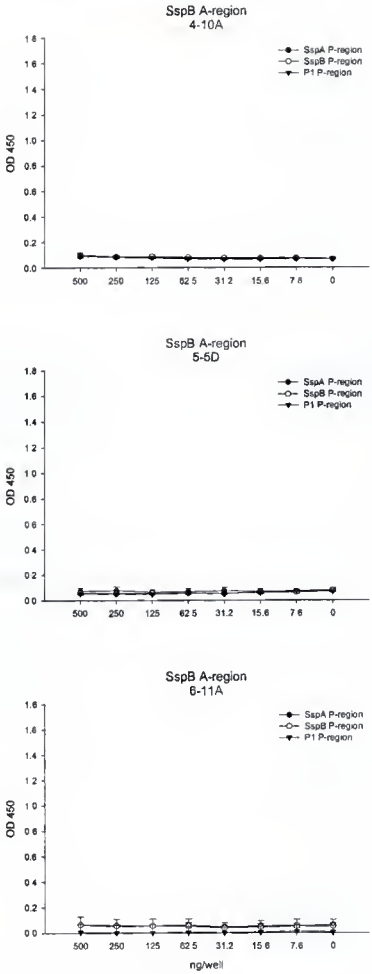

Figure 17. Restoration of epitopes by the interaction of various antigen I/II A-regions with the immobilized A-region of SspA. Panel titles indicate the source of immobilized P-region and the mAb tested. Legend indicates the source of the overlaid P-regions.

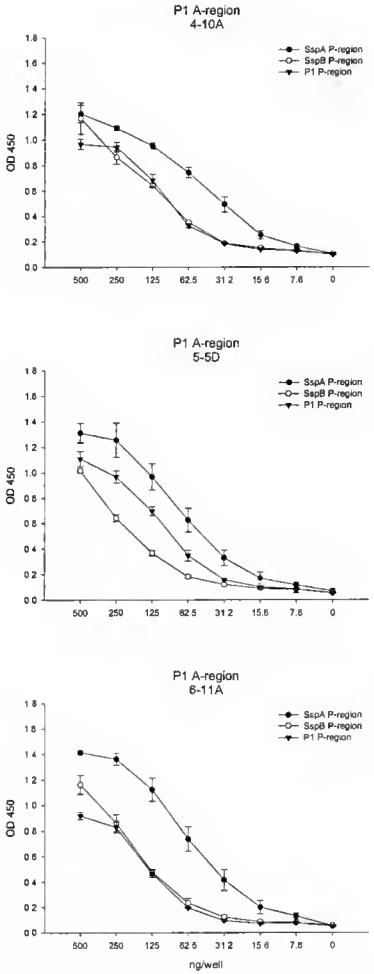

Figure 18. Restoration of epitopes by the interaction of various antigen I/II A-regions with the immobilized A-region of SspA. Panel titles indicate the source of immobilized P-region and the mAb tested. Legend indicates the source of the overlaid P-regions.

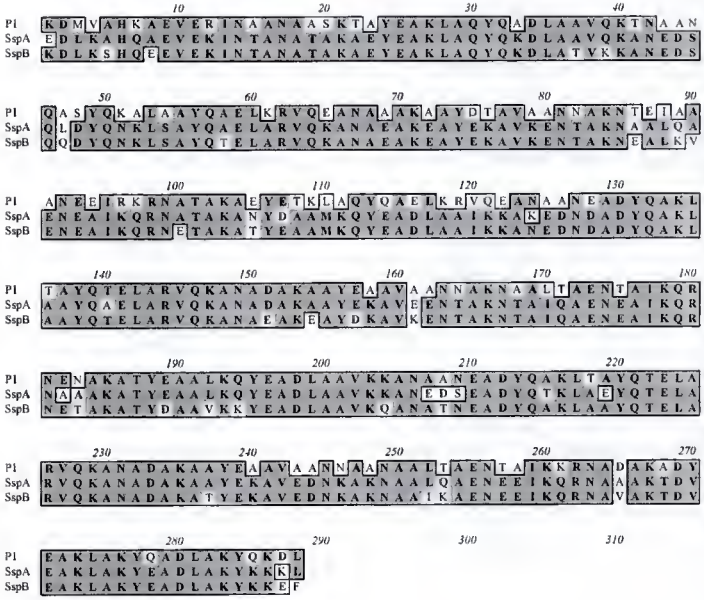

Figure 19. CLUSTAL W alignment of the A-regions of P1, SspA, and SspB. Dark grey shading indicates identity. Light grey shading indicates similarity.

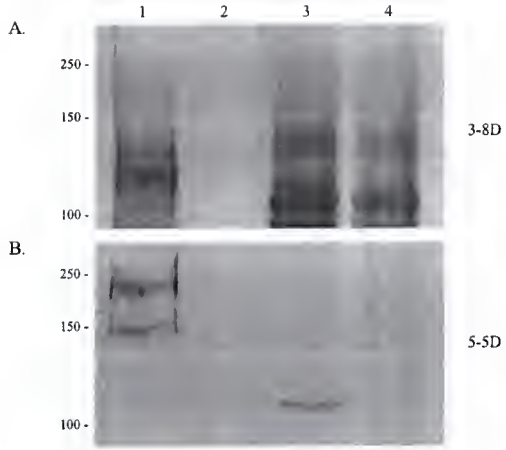

Figure 20. Western immunoblot of chimeric P1 containing the A-regions of SspA and SspB. Whole cell lysates of *E. coli* DH5α harboring plasmids encoding P1 containing with the A-regions of SspA and SspB. Lanes contain P1 (1), P1ΔA (2), P1ΔA + A-region of SspA (3), P1ΔA + A-region of SspB (4). Panel A was reacted with the A-region specific mAb 3-8D. Panel B was reacted with the A- and P-region dependent mAb 5-5D.

Figure 21. Surface expression of *S. gordonii* SspA and SspB in *S.* mutans PC3370. Whole cell dot blot of P1-deficient *S. mutans* PC3370 complemented with plasmid-encoded P1, SspA, and SspB. Surface expression was traced with mAb 5-5D.

Figure 22. Demonstration of lack of ability of heterologous A-regions to restore surface expression of P1ΔA in PC3370. Whole cell dot blot of PC3370 harboring vector alone (1) and plasmids expressing P1 (2), P1ΔA (3), and P1 containing the A-regions of SspA (4) and SspB (5). Surface expression of P1 was detected with mAb 5-5D.

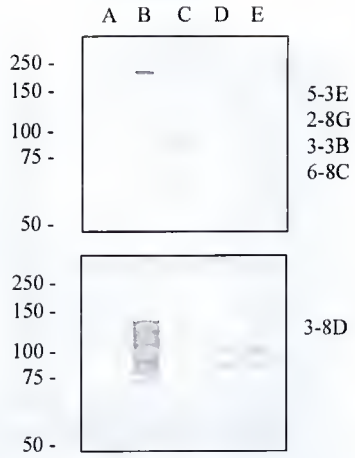

Figure 23. Western immunoblots of cell lysates of PC3370 harboring vector alone (A), and plasmids encoding P1 (B), P1ΔA (C), P1ΔA + SspA A-region (D), and P1ΔA + SspB A-region. P1 was detected with C-terminals specific mAbs (upper panel) and A-region specific mAb 3-8D (lower panel).

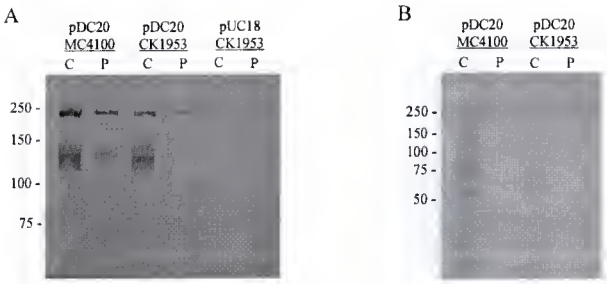

Figure 24. Western immunoblot of cytoplasm (C) and periplasm (P) fractions of *E. coli* MC4100 (wild-type) and CK1953 (Δ*secB*) harboring pDC20 (P1). P1 was traced with mAbs 5-3E, 2-8G, and 6-8C in panel A. In panel B, β-galactosidase was traced with a rabbit polyclonal antibody. Migration of molecular weight standards are indicated in kilodaltons.

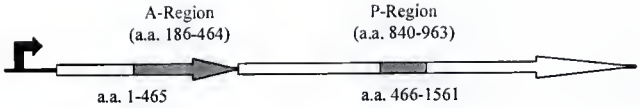

Figure 25. Schematic representation of discontinuous P1. Black arrow represents the *spaP* promoter. N-terminal open reading frame (ORF) expresses residues 1-465 including the A-region (shaded). C-terminal ORF expresses residues 466-1561, which includes the P-region (shaded).

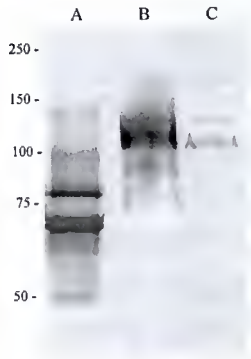

Figure 26. Western immunoblot of P1 fragments expressed from pTS30 in *E. coli* and traced with mAbs 3-8D (A), 4-10A (B), and 5-3E (C). Migration of molecular weight standards are indicated in kilodaltons.

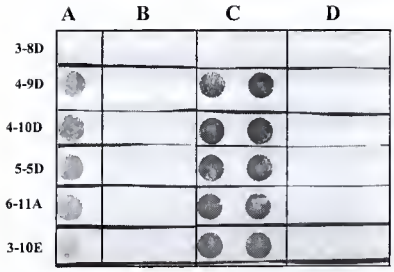

Figure 27. Whole cell dot blot of *S. mutans* NG8 (A) and PC3370 harboring pDL289 vector control (B) pMAD encoding P1 (C), and pTS31 encoding discontinuous P1 fragments (D). Surface expression of P1 polypeptides was traced with the indicated anti-P1 mAbs.

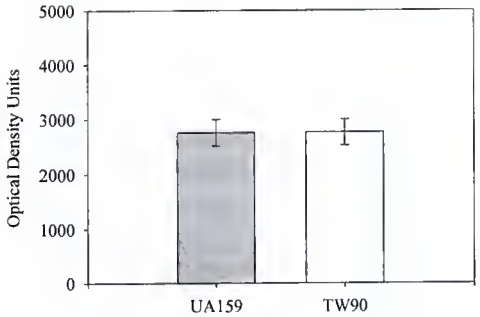

Figure 28. P1 surface expression levels of *S. mutans* UA159 and TW90 (*ropA*- mutant) at early log stage traced with mAb 4-10A as measured by densitometry.

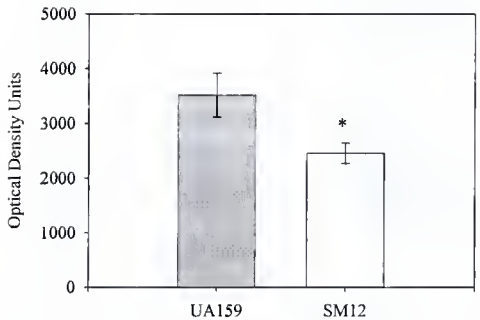

Figure 29. P1 surface expression levels of *S. mutans* UA159 and SM12 (DnaK-deficient) at early log phase traced with mAb 4-10A as measured by densitometry. No difference was detected at stationary phase ([n = 12]* statistically significant, P<0.0001. Significance was determined by student's t-test.)

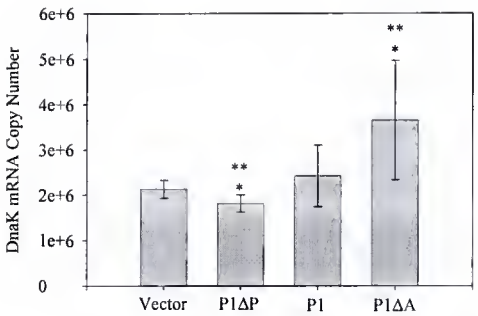

Figure 30. Real-Time PCR quantification of *dnaK* mRNA from *S. mutans* PC3370 harboring the pDL289 vector alone and expressing P1ΔP-region, full-length P1, and P1ΔA-region. ([n = 36]* statistically significant compared to vector, P<0.005. ** statistically significant compared to P1, P<0.05. Significance was determined by student's t-test.)

CHAPTER 4
DISCUSSION AND CONCLUSIONS

Dental caries is one of the most prevalent oral diseases worldwide, affecting 60-90% of schoolchildren and the majority of adults. In the United States, dental caries is the most common chronic childhood disease with 78% of 17 year olds having at least one cavity or filling [130] and, according to the U.S. Department of Health and Human Services, it is estimated that over $84 billion dollars is spent annually on dental treatment and caries prevention in the United States alone. While advances in dental care and caries prevention has reduced the incidence of caries in developed countries, the incidence of caries worldwide has remained unchanged for the past 20 years [131].

A major contributing factor to the decline of caries in developed countries was the introduction of fluoride to water and toothpaste. Unfortunately, in developing countries, where the incidence of caries is on the rise, fluoridated community water is commonly not a viable option. Although fluoridated water reaches 60% of the U.S. population, more than 90% of toothpastes contain fluoride, and processed food and beverages often contain fluoride, the reduction in caries incidence has been uneven across the general population in the United States. The majority of the disease is now being borne by a disproportionately small segment of the population; those of low socioeconomic status, low education, and lack of access to dental care [132-134], in essence a mirror of the populations in developing countries. The inability to manage caries in this subpopulation of the United States, where the dentist to population ratio is better than 1:2000 people,

illustrates the improbable task of preventing caries in developing countries, such as in Africa where the ratio is1:150,000. A better understanding of the virulence factors and/or targets of protective immunity in *S. mutans* could lead to preventative measures that could help overcome the lack of resources, education, and infrastructure that is currently required for caries prevention.

The major surface protein P1 of the cariogenic organism, *S. mutans*, is a multifunctional adhesin and plays a role in the attachment of the bacterium to the tooth surface. P1 shares similarities to virulence factors of several other bacterial species, including the fibronectin binding proteins of *S. aureus* and *S. pyogenes* [65, 66], and the pneumococcal surface protein (PspA) of *S. pneumonia* [68]. P1 has been identified as a target for protective immunity and has been studied as a potential antigen candidate for an anti-caries vaccine [135]. It has also been used for the production of mAbs that are currently being investigated for their ability to modulate the immune response in mice that are challenged with mAb-*S. mutans* or mAb-P1 complexes [136]. Also, with little known about the maturation and translocation of Streptococcal surface proteins, P1 is a potential model for studies in these areas.

The goal of this research was to further our understanding of the structure and antigenic properties of this large and complex molecule with an emphasizes on identifying intramolecular interactions, the contribution of intramolecular interactions to structure, stability, and translocation of P1, and to begin to identify chaperones that contribute to P1 maturation and translocation.

Identification of an Intramolecular Interaction within P1

Previously, by process of elimination using truncated P1 polypeptides, the central region of P1 was determined to contribute to the epitopes of six of eleven anti-P1 mAbs

(4-9D, 4-10A, 5-5D, 6-11A, 3-10E, and 1-6F) [120]. It was additionally shown that deletion of the P-region of P1 (P1ΔP) abrogated the binding of four of the eleven mAbs (4-10A, 5-5D, 6-11A, and 3-10E) and that none of these antibodies recognized a subcloned P-region peptide suggesting that their epitopes were complex and conformational. Surprisingly, although P1ΔP retained its N-terminal signal sequence and C-terminal cell wall anchoring motif it was unstable in *S. mutans* and not translocated to the cell surface [54]. Proline-rich regions have been shown to be involved in both protein-protein interactions and intramolecular chaperone-like interactions. The initial objective of these studies was to identify interactions between the P-region and other regions of P1.

Work by Rhodin et al. [123] on the characterization of the mAb 6-11A epitope further defined regions of P1 that were required for reactivity of the 6-11A. Analysis of several P-region spanning P1 subclones revealed that in addition to the P-region (a.a. 819-1017), residues N-terminal of D465 also contributed to the reactivity of mAb 6-11A. In addition, the crystal structure of the P1 variable region suggested that the A- and P-regions may be in close proximity [30], and it was reported that a polypeptide containing the P-region bound to the N-terminal third of P1 [124]. Based upon these reports, the A-region was examined to determine whether it contributed to a complex structure by association with the P-region.

The initial experiment was to examine the effects of the removal of the A-region from P1. Therefore, a *spaP* gene lacking the A-region (a.a. 179-466) was constructed by PCR and cloned into pUC18, creating pTS20. The construct was engineered with a silent mutation that produced a unique *Sfo*I restriction site that would later be used to insert

heterologous A-regions. The insertion of the *Sfo*I site dictated the exact residues that were deleted. Deletion of the A-region resulted in a loss of reactivity of five of eleven of the anti-P1 MAbs (3-8D, 4-9D, 4-10A, 5-5D, and 6-11A), 3 of which are also dependent upon the presence of the P-region (4-10A, 5-5D, and 6-11A) (see Figure 2). This suggests that the epitopes of these three antibodies are complex and composed of portions of discontinuous segments or that an interaction between the regions results in conformational epitopes being produced within one or both of the regions. Reactivity of Mabs 5-3E, 2-8G, 3-3B, and 6-8C, which are specific to the C-terminal of P1, confirmed that the deletion of the DNA encoding the A-region did not disrupt the reading frame. The Western immunoblot also shows that like P1ΔP, P1ΔA is stably expressed and detectable in *E. coli*.

The presence of internal proline-rich regions has been associated with aberrant migration of streptococcal and staphylococcal proteins on SDS-polyacrylamide gels. The molecular mass of P1 has been predicted to be ~166 kDa, although the protein migrates with an apparent molecular mass of ~185 kDa by SDS-PAGE. Interestingly, P1ΔP and P1ΔA run at their predicted sizes of 152 kDa and 135 kDa, respectively, suggesting that an interaction between the A- and the P-regions may contribute to anomalous migration of P1 by SDS-PAGE. On a sided note, the abberant migration of P1 was observed even after "denaturing" in 8 M urea and SDS-PAGE at both 4°C and 60°C.

With the data from the A-region deletion indicating that the A- and P-regions contribute to the same epitopes, it was of interest to determine if these regions were, in fact, capable of interacting. An interaction between recombinant polypeptides corresponding to the isolated A-region (a.a. 186-469) and P-region (a.a.819-1017) was

examined by ELISA. A-region polypeptide was incubated with immobilized P-region and A-region binding was detected with anti-P1 MAb 3-8D, which recognizes an epitope contained entirely within the alanine-rich repeats. The ELISA revealed a direct, dose-dependent, interaction (see Figure 6).

The required simultaneous presence of both the A- and P-regions for the binding of mAbs 4-10A, 5-5D, and 6-11A to P1 and the ability of recombinant A- and P-region polypeptides to interact suggested that the interaction of these regions could contribute to the epitopes that are recognized by these mAbs. To further analyze the characteristics of the epitopes recognized by these mAbs, ELISA were performed with A- and P-region polypeptides to determine whether binding of mAbs 4-10A, 5-5D, or 6-11A was restored upon interaction of these two discontinuous domains. Interestingly, mAbs 5-5D and 6-11A reacted considerably better when the A-region was applied to immobilized P-region rather than visa versa, while MAb 4-10A displayed no apparent preference (see Figure 7). This would suggest that the contact residues for MAbs 5-5D and 6-11A are largely contained within the A-region while both regions may contain residues required for the MAb 4-10A epitope. Since, all three of these mAbs bind to P1 on the surface of *S. mutans*, these results indicate that the A- and P-regions interact in the context of the whole molecule in its native conformation.

Due to the possibility that the interaction of the A- and P-regions may be an artifact of being immobilized to the ELISA plate, a competitive inhibition ELISA was performed to assess the interaction of the A- and P-regions in solution. Since it was shown that an interaction of the A- and P-region is required for the binding of MAb 4-10A, recombinant A- and P-region polypeptides were used alone and in combination to inhibit the binding

of MAb 4-10A to immobilized P1. As can be seen in Figure 8, neither the A-region nor the P-region individually inhibits MAb 4-10A binding to P1, however the combination of both polypeptides does. This indicates that the A- and P-regions are capable of interacting in solution.

In an effort to establish the stoichiometry of the A- and P-region interaction, a variation of the Job Plot was performed. The Job Plot, or continuous variation, consists of mixing two binding partners, or an enzyme and substrate, at various molar ratios while holding the total concentration constant and then recording a measurable change. While continuous variation is normally performed in solution, due to the use of antibodies to measure the A- and P-region interaction, the A- and P-region polypeptides had to be immobilized to an ELISA plate to afford the removal of excess unbound antibody. Since MAb 4-10A was the tool used to measure the A- and P-region interaction, the stoichiometry that was determined would actually be that required for the formation of the MAb 4-10A epitope. According to the assay, the epitope of mAb 4-10A consists of a 1:1 ration of A-region to P-region (see Figure 9).

Although antigen I/II proteins are highly conserved, the functional properties of individual members of this family of proteins differ. *S. gordonii* possesses two antigen I/II proteins, SspA and SspB, which have been well characterized. Several functional differences between these two proteins have been identified, including coaggregation with other oral flora and interaction with type 1 collagen [137, 138]. P1 is closer to SspA in homology, 67% identity versus 57% with SspB. Specific amino acid residues that are not in P1 have been identified to be important for SspB binding to *Porhromonas gingivalis* [139] and interactions of SspB and P1 with salivary agglutinin also differ

[140]. Recent studies focusing on the A-regions of SspA, SspB, and P1 have also identified structural and functional variation. It was reported that the A-regions of P1 and SspA bound to salivary agglutinin but that the A-region of SspB did not. In addition, structural analysis suggested that the A-region of SspB is less stable than that of SspA and P1, both at high temperature and low pH. It should be noted that the A-regions of SspA and SspB exhibit approximately 87% primary sequence identity with one another while the A-regions of SspA and P1 only share 70% (see Figure 19) [35, 36, 116, 141].

In light of the similarities and differences reported between the A-regions of SspA, SspB, and P1, it was of interest to see if the A- and P-regions of these *S. gordonii* proteins interacted with one another as well as with the A-and P-regions of P1. Again, interaction between these regions was examined by ELISA and a dose-dependent interaction was observed with mAb 3-8D reacting to A-region polypeptides binding to immobilized P-region polypeptides (see Figure 11). The results indicate that the A-region of P1 interacts more strongly with all of the P-regions than either of the *S. gordonii* polypetides and that the SspB A-region is the weakest binder, which follows the trend of P1 being more like SspA.

The contribution of the P1 A-region and P-region interactions to native structure as determined by epitope restoration and the ability of the heterologous interaction of A- and P-regions led to the examination of epitope restoration by the interaction of the heterologous A- and P-regions. As before, ELISA was used to detect the restoration of epitopes for mAbs 4-10A, 5-5D, and 6-11A by every combination of P1, SspA, and SspB A- and P-region interactions in which eith the A- or P-region was immobilized. The results demonstrated that the epitopes recognized by these three A- and P-region

dependent mAbs were restored regardless of which P-region interacted with the immobilized A-region from P1 (see Figure 18). While when P-regions were interacted with either of the *S. gordonii* A-regions, only the P-region of P1 restored any mAb reactivity, which was for mAb 5-5D and at a low level (see Figures16 and 17). Additionally, the A-region of P1 was able to restore all of the epitopes when interacted with any of the immobilized P-regions and the A-region of SspA was able to restore the mAb 5-5D epitope when interacted with immobilized P1 P-region (see Figure 15). And lastly, the interaction of the A-region of SspB with any immobilized P-region failed to restore epitopes.

Comparing the results of the A- and P-region interactions to the reactivity of mAbs 4-10A, 5-5D, and 6-11A against full-length SspA and SspB as examined by Western immunoblot was interesting (see Figure 12). While mAb 5-5D reacted to both full-length SspA and SspB, its epitope was not restored by the interaction of the SspA and SspB A- and P-regions. The same held true for mAb 4-10A which bound to full-length SspB and weakly reacted to SspA. To summarize, although mAbs 4-10A and 5-5D bound to full-length SspA and SspB, their epitopes were not restored by interactions of the A- and P-regions of these proteins unless one of the interacting regions was from P1.

Analysis of P1 Translocation and the Contribution of the Alanine- and Proline-Rich Regions

All life depends upon the targeting of newly synthesized proteins to their site of action. During transit to its destination, a protein must avoid a variety of hazards such as malfolding, aggregation, and degradation and may be required to pass through one or more membranes, known as translocation. Protein translocation has been extensively

studied in *E. coli* and the models established through this research are believed to be representative of all bacterial cells [142-144] but more recently, *Bacillus subtilis* has become the model for Gram-positive bacteria. Proteins that are targeted for translocation across the cytoplasmic membrane contain an N-terminal signal sequence [145] that generally contains positively charged residues followed by 15 to 20 hydrophobic residues[146, 147], which are usually removed during or shortly after translocation. Signal peptides can be classified by the type of signal peptidase that is responsible for their proteolytic processing and these classifications can be used to predict the translocation pathways [148]. Based upon surveys of signal peptides in the genomes of *B. subtilis* and several other Gram-positive bacterium, it is predicted that most extracellular proteins in these organisms are secreted via the Sec-translocase [149-151] In *Bacillus subtilis*, there are four predicted protein transport pathways; (i) the Sec-dependent pathway, (ii) Twin–arginine translocation (Tat), (iii) ABC transporter-dependent secretion pathways, (iv) and a pseudopilin-specific export pathway. A survey of the *S. mutans* UA159 genome failed to reveal any homologues of the Tat machinery or Tat signal peptides.

In addition to the requirement for MAb reactivity, the simultaneous presence of both the A- and the P-regions appear to be required for P1 stability in *S. mutans*. Analysis of mRNA encoding P1ΔA, like P1ΔP [54], demonstrated that the internally deleted *spaP* gene was transcribed at levels equivalent to the wild-type *spaP* gene (see Figure 4). Differences in *dnaK* mRNA levels in *S. mutans* harboring plasmids encoding P1, P1ΔA, and P1ΔP also suggested that the P1ΔA was being translated. However, no P1ΔA was detected in the cytoplasm, on the cell surface (see Figure 3), nor in the culture

liquor. While P1ΔP contains a deletion of 170 residues and P1ΔA lacks 287 residues, there are examples of stable antigen I/II polypeptides that, when compared to P1, are lacking large segments of the molecules. The antigen I/II protein expressed by *S. intermedius*, Pas, lacks ~270 residues from the A-region and ~80 residues from the P-region [152]; Paa from *S. cricetus* possesses an additional ~139 residues in the A-region and ~39 residues less in the P-region [152]; and *S. mutans* GS-5 expresses a PAc molecule lacking the C-terminal ~400 residues [153]. The A-region of P1 consists of three-82 residue repeats and the P-region consists of three 39-residue repeats and both Paa and Pas retain repeats in both regions. Not all internal deletions in P1 result in the apparent level of instability seen in P1ΔA and P1ΔP. Rhodin et al. constructed a P1 construct lacking residues 84-190 which was detectable in *S. mutans* PC3370, but was not translocated to the surface (unpublished). This suggests that the A- and P-regions may contain inherent structural information, possible chaperone binding sites, or perhaps possess chaperone-like activities that are critical to P1 stability. A proline-rich region has been implicated as an intramolecular chaperone by Wang et al. [79]. The central proline-rich region of the *Limulus* secreted serine protease, Factor C, was shown to be required for secretion of the molecule. Their data suggested that the correct folding of the molecule C-terminal of the proline-rich region was dependent upon the presence of the proline-rich region and that the lack of secretion was due to malfolding.

To fully understand the role of the A- and P-regions in P1 translocation, identifying the molecule's route of translocation is necessary. There is no experimental data that identifies the secretion pathway employed by P1 or antigen I/II-like proteins. Cell wall anchoring of P1 and PAc is mediated by the transpeptidase sortase [85, 154] and sortase

anchored proteins are presumed to be translocated via the sec translocase [86]. As detailed in the introduction, the Sec-dependent secretion pathway has been thoroughly studied in *E. coli* and the characteristics of the pathway are presumed to be conserved for all bacteria. In *E. coli*, the Sec-translocase consists of a structure composed of several proteins including the ATPase SecA, which provides the energy for translocation [87]. Current literature identifies two major pathways that a nascent protein destined for the Sec-translocase would be transported upon, the signal recognition particle (SRP) pathway and the SecB pathway.

The SRP pathway is involved in co-translational protein secretion. The SRP recognizes and binds to the signal peptides of nascent polypeptides as they emerge from the ribosome [89]. Binding of the SRP stalls translation and targets the SRP-ribosome complex to the SRP receptor, FtsY [90, 91]. The SRP-ribosome-FtsY complex is then targeted to the Sec-translocon where the ribosome docks and the protein is co-translationally translocated across the membrane [87].

The cytoplasmic chaperone SecB targets preproteins to the Sec-translocon for post-translational translocation. SecB binds to nascent and full-length proteins as they emerge from the ribosome [92]. SecB interaction prevents premature folding of the preprotein and delivers it to the Sec-translocon in a secretion-competent state. Binding of the SecB-protein complex with SecA results in the transfer of the preprotein to SecA and the release of SecB [93]. The protein is subsequently translocated across the membrane through the Sec-translocon [94].

Due to the faint expression of P1ΔP and the undetectable expression of P1ΔA in *S. mutans*, *E. coli* was used to begin to examine P1 secretion. P1ΔP and P1ΔA are stable

and detectable, albeit at reduced levels, in whole cell lysates of *E. coli* by Western immunoblot. Analysis of periplasmic extracts by Western immunoblot revealed that P1 was secreted into the periplasm, but P1ΔP and P1ΔA were not. This suggests tbat while the A- and P-regions are apparently not required for stability in *E. coli*, the regions are required for secretion. If a lack of chaperone interaction with the deletion recombinant proteins results in the lack of secretion, perhaps a similar lack of interaction also favors degradation of the molecules in *S. mutans*. It is known that secretion incompetent proteins are subject to rapid turnover, which is a likely scenario when these constructs are expressed in *S. mutans*. With such a rapid turnover in *S. mutans* and considering the time required for lysis of the bacterium, pulse-chase experiments to determine the half-life of these deletion proteins in Streptococci are currently technically improbable.

The SRP pathway has been identified in both gram-negative and gram-positive bacteria. In *B. subtilis*, numerous homologs of the general secretory pathway components have been identified. However, as is the case with *S. mutans*, no SecB homolog has been identified, but *B. subtilis* has been found to possess a functional ortholog, CsaA, which has been shown to have partially overlapping binding characteristics [95-97]. As previously stated, the SRP is essential for viability in *E. coli* and it was assumed to be the case in all organisms. However, it has been demonstrated that *S. mutans* is viable without SRP [98] and P1 is translocated and expressed on the cell surface in its absence (unpublished). This would suggest that if P1 secretion is Sec-dependent, the targeting pathway should likely be SecB-like and require a functional SecB ortholog. To examine the possibility of a role for SecB in P1 secretion, P1 was expressed in a SecB-negative *E. coli* mutant, CK1953. P1 was shown to be stable and

secreted into the periplasm in CK1953. This suggests that, in *E. coli*, if P1 is secreted via the Sec-pathway, it is associating with an alternative chaperone to SecB, a functional equivalent, or that it may be able to use the SRP pathway. Recent evidence suggests that in the *S. mutans* SRP- mutants, the protein YidC may be involved in a compensatory pathway (unpublished), illustrating the ever-evolving complexity of bacterial protein secretion pathways.

The demonstrated interaction of the A- and P-regions combined with the apparent requirement of their interaction for the restoration of structure as evidenced by epitope restoration led to the construction of a plasmid, pTS31, which expressed P1 as two fragments. The purpose of this construct was to examine whether the expression of the A- and P-regions on separate polypeptides would be sufficient for translocation of the polypeptides across the cell membrane. Precedence for *trans* complementation with an intramolecular chaperone resulting in protein secretion and function was demonstrated with *P. aeruginosa* LasB [126]. The recently discovered twin-arginine-translocation (TAT) pathway transports folded proteins across cell membranes [155] and although a survey of the *S. mutans* genome did not reveal any homologous TAT genes or TAT signal sequences, the translocation characteristics of the P1 derivatives and the interaction of the A- and P-regions suggested the possibility of a partial folding requirement prior to translocation.

In *E. coli*, both the N-terminal A-region and C-terminal P-region containing fragments could be easily detected in the cell lysates by Western immunoblot (see Figure 26). However, in *S. mutans*, only the A-region containing fragment was detected in the cell extracts. In addition the N-terminal fragment could be detected in the *S. mutans*

culture supernatant. These results indicate that the amino-terminal signal sequence is sufficient for the stable expression and translocation of a P1 polypeptide consisting of the first 480 amino acids. Also, that the A-region fragment expressed in *trans* is not sufficient to protect the apparently unstable carboxy-terminal 1081 amino acids in *S. mutans.* These results did not reveal any details of P1 translocation that contradict the presumed use of the Sec translocase.

It appears from the above results that the A-and P-region must reside within the same molecule for the stable expression of P1. To begin to identify which residues within the A-region are important for P1 stability, structure, and translocation, the DNA encoding the A-regions from *S. gordonii* SspA and SspB was ligated into the site of the A-region deletion in P1ΔA. The chimeric P1 proteins were expressed in both *E. coli* and *S. mutans* PC3370 and restoration of stability and structure, as determined by epitope restoration, was examined by Western immunoblot. In *E. coli*, the immunoblots indicated that some native structure was restored by the A-region of SspA as suggested by restoration of the mAb 5-5D epitope (see Figure 20). However the A-region of SspA did not restore 4-10A or 6-11A binding and the insertion of the A- region of SspB did not restore any of the A- and P-region dependent epitopes. These results agree with the previously demonstrated restoration of the mAb 5-5D epitope by the interaction of the A-region of SspA and the P-region of P1.

Full-length SspA and SspB were translocated to the surface of PC3370 indicating that there was no inherent instability when expressed in *S. mutans* (see Figure 21). The introduction of the heterologous A-regions did not restore surface expression of P1 in PC3370 (see Figure 22). In addition, no full-length chimeric P1 proteins were detected in

lysates of PC3370 by Western immunoblots. However, break down products were detected by the A-regions specific mAb 3-8D (see Figure 23). As seen previously with the expression of the discontinuous P1 fragments, the N-terminal appears to be more stable than the C-terminal of P1. The lack of stability and translocation of the chimeric P1 proteins in PC3370 suggests that information intrinsic to the P1 A-region is required for P1 stability and that, although highly conserved, the structural information provided by the A-regions of the antigen I/II proteins may be protein specific.

Of the 288 amino acid residues in the A-regions that were swapped, the A-region of P1 differs from the A-region of SspA at 86 residues although 34 of those have similar properties and with SspB at 94 residues with 45 being similar (see Figure 19). The differences between the A-regions in SspA and SspB are at 36 residues with 19 being similar. It is unlikely that a single residue is responsible for the difference between the partial restoration in structure that is seen with the SspA A-region and complete native P1 structure. However, by subtracting those residues that SspB has in common with P1 from the residues that differ between SspA and P1, the field of candidates can be narrowed to 24 residues with 14 being similar.

These results can be used to define target residues for potential site directed mutagenesis in future studies. In addition, as previously stated, the *S. intermedius* Pas protein is missing 2/3 of the A-region and is still stable which illustrates that there is no global requirement for the entire A-region. Future experiments involving the insertion of A-region segments into P1ΔA may also elucidate a minimum requirement for P1 stability.

Involvement of RopA (Trigger Factor) and DnaK in the Maturation and Translocation of P1

Signal peptides are necessary and sufficient for protein translocation if the protein can be maintained in a secretion-competent state, which for most proteins is an unfolded state. To maintain this export competent conformation, precursor proteins interact with cytoplasmic chaperones. Chaperones do not recognize signal peptides, but they play an essential role in the transport and translocation of extracellular proteins by binding to and maintaining their protein substrates in a loosely folded conformation that is required for translocation across the membrane [156]. To begin to characterize the chaperones involved in the translocation and maturation of P1, the chaperones RopA and DnaK were examined.

The bacterial heat shock protein DnaK is involved in a wide-variety of cellular processes ranging from assisting in protein folding to targeting a protein for degradation. The common function of DnaK is to bind to short hydrophobic regions of polypeptides that are generally not exposed in properly folded proteins. DnaK binding to these regions prevents protein aggregation and halts folding. Depending upon the cellular conditions, the protein may be transferred to more specialized chaperones for refolding or destined for degradation. Several studies have shown that DnaK can also maintain the translocation competence of presecretory proteins which is also the role of SecB [157-160]. DnaK is structurally unrelated to SecB and its binding to polypeptides is ATP-dependent and is regulated by co-chaperones [161, 162]. In addition, the substrate specificity and for each chaperone differs considerably [161-164]. Evidence suggests that chaperones have overlapping functions in the protein export pathway. The

functional redundancy of chaperones may explain why SecB in *E. coli* and SRP in *S. mutans* are not essential for cell viability.

The first chaperone or folding catalyst that interacts with nascent presecretory proteins is the peptidyl prolyl isomerase trigger factor [102, 165]. Trigger factor is an abundant cytoplasmic protein which catalyzes *trans* to *cis* prolyl bond isomerization during the refolding of denatured proteins. In *E. coli*, trigger factor associates with the 50s subunit of the ribosome [166] and it was found to interact with a wide variety of nascent proteins both *in vitro* as well as *in vivo* [89, 102, 103, 165, 167]. *In vivo* studies present evidence that trigger factor has a role in the cytosolic folding pathway together with the cytoplasmic chaperone DnaK [103, 167]. DnaK was found to interact under non-stress conditions with nascent chains with of 30 kDa or longer. Deletion of the non-essential gene encoding trigger factor resulted in the doubling of the number of nascent chains interacting with DnaK. Under these conditions DnaK interacted with much shorter nascent chains, suggesting that trigger factor is the first protein that interacts with the nascent chains as soon as they emerge from the ribosome.

Several recent studies have revealed that in addition to isomerase activity, trigger factor has chaperone activity. Trigger factor prevents the aggregation of proteins either in combination with GroEL-GroES or alone [168] and is necessary for the breakdown of abnormal proteins [169]. Moreover, refolding of GAPDH was shown to be assisted by the chaperone function of trigger factor [170] and the isomerase activity of trigger factor has been shown not to be required for the folding of newly synthesized cytoplasmic proteins in *E. coli* [106].

Additionall, a role for trigger factor in secretion was demonstrated by Beck et al. [171] who suggested that trigger factor has a role as a decision maker and directs presecretory proteins into the chaperone based targeting pathway. However, trigger factor has not been found to be essential for secretion of any known protein in *E. coli* or *B. subtilis* nor is it essential for cell viability [167, 172, 173]. In *S. pyogenes*, however, trigger factor (RopA) is essential for the secretion and maturation of the cysteine protease, SpeB. Lyon et al. demonstrated that in the absence of RopA, the nascent protease polypeptide was not targeted to the secretory pathway. While an in-frame deletion of the PPIase domain within RopA resulted in a secreted but enzymatically inactive protease, suggesting that trigger factor has an additional role in protease maturation [174]. It was subsequently determined that a single proline, P78, was the target of the PPIase activity that was required for maturation of the protease [107]. It is interesting that the lack of isomerization can send the protease into an alternative folding pathway which results in a malfunctioning enzyme, yet not appear to effect its secretion. In *S. mutans*, RopA was recently found to be involved in stress tolerance and biofilm formation [112]

The involvement of RopA in the expression of functional P1 in *S. mutans* was examined using an adherence assay and analyzed with a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) as described in [128].. The binding of *S. mutans* to salivary agglutinin has been shown to be mediated by P1 and the binding can be inhibited by the P1-specific Mab 4-10A, but not by the P1-specific Mab 6-11A [33]. Adherence of the *ropA*-negative *S. mutans* TW90 to salivary agglutinin was substantially lower (< 50%) than that of the wild-type UA159 and the inhibition of adherence of both UA159 and TW90 by the

addition of Mab 4-10A indicated that the adherence was P1 mediated. An obvious cause of the reduction in adherence would be a reduction in surface expressed P1; however, whole cell dot blots indicated that this was not the case. TW90 and UA159 expressed equivalent levels of P1 on their surfaces at both time points examined, early-log and stationary phase (see Figure 28). These results indicate that RopA is not required for the secretion of P1, but suggest that it is required for the maturation of P1 to a fully functional adhesin. Whether the effect is due to the lack of a direct interaction between RopA and nascent P1 or an indirect effect through intermediates is unknown and the subject of future studies.

Since DnaK is required for *S. mutans* viability, the effect of DnaK on the surface expression of P1 was examined in *S. mutans* SM12, which expresses only 5% of wild-type levels of DnaK. Whole cell dot blots of SM12 harvested at early-log phase expressed significantly reduced levels of P1 on the surface compared to wild-type UA159 (see Figure 29). However at stationary phase the levels of surface P1 were equivalent. These results do not necessarily indicate a direct interaction of DnaK with P1 as the reduced surface expression may be a general reduction in all protein translocation. Additionally, like with RopA, any observed effects may be due to required DnaK interactions elsewhere in the secretion pathway. At the stationary growth stage, P1 surface expression in the down-regulated DnaK mutant is equivalent to the wild-type suggesting that the continuous, but reduced, translocation of P1 eventually catches up to the wild-type levels. Whether the reduced level of translocation is due to a direct effect of DnaK on P1 or is due to an effect on the secretion pathway components is still to be determined.

Quantitative Real-Time PCR was used to measure the effects of the presence of P1, P1ΔA, and P1ΔP on *dnaK* mRNA levels in *S. mutans* PC3370 (see Figure 30). Statistical analysis of the data revealed significant differences between the levels of *dnaK* mRNA in PC3370 harboring the vector only verses PC3370 expressing P1ΔA and P1ΔP. Oddly, the level of mRNA in the cells expressing P1ΔP was lower than in the vector only control. The highest level of *dnaK* mRNA was in PC3370 expressing P1ΔA.

In *E.* coli, transcription of *dnaK* is initiated by a σ^{32} promoter which DnaK is involved in regulating by interacting with the σ^{32} subunit of RNA polymerase. It was proposed that the presence of denatured proteins acts as a sink for DnaK thus freeing σ^{32} thus initiating *dnaK* transcription[175]. *DnaK* in *S. mutans*, on the other hand, is transcribed from a σ^{A}-type promoter [176] and is negatively regulated by HrcA [177].

Although neither P1ΔA nor P1ΔP could be detected in PC3370 using immunological methods the effect of their expression on *dnaK* mRNA levels would suggest that they were indeed translated. The difference in *dnaK* mRNA levels in the presence of P1ΔA verses P1ΔA may be a circumstance of protein stability in that P1ΔP might be turned over more rapidly than P1ΔA and that DnaK was not involved in the process. It is also plausible that the P-region in the malfolded P1ΔA acted as a sink for DnaK which resulted in upregulation in a manner of transcriptional control that is similar to that seen with the *E. coli* σ^{32}. Further research will be needed to determine if the difference in expression levels correlates to differences in DnaK binding to P1 and its derivatives.

Conclusions

The goal of this research was to provide insight into the contribution of the proline-rich region to the structure, translocation, and antigenicity of P1. The analysis of the P-

region and its interaction with the A-region has revealed information regarding the structure of the molecule and the complexity of the epitopes that are recognized by anti-P1 mAbs. These studies have also shown a requirement of these regions for P1 stability and translocation to the cell surface of *S. mutans* and to the periplasm of *E. coli.* In addition, it has established *E. coli* as a viable tool for future P1 translocation studies. The results of this research have implications for understanding surface localization of virulence factors in pathogenic microorganisms and for understanding how the protein structure of a vaccine antigen contributes to recognition by antibodies.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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